Effects of development temperature on the reproductive anatomy and behaviour of female *Callosobruchus maculatus*

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Abstract

Primary reproductive traits (those traits directly associated with sexual reproduction) have evolved both rapidly and divergently. Despite considerable advances in our understanding of the mechanisms that drive the evolution of these traits, we still have a very limited understanding of how male and female traits interact and how environmental factors influence the expression of these traits. Previous research has shown developmental temperature to affect the ejaculatory characteristics of male *Callosobruchus maculatus*, which subsequently affected copulatory behaviour and the outcome of sperm competition. However, given there is some evidence that both copulatory behaviour and the outcome of sperm competition are a product of male-female interactions, I here examine the effect of developmental temperature on the expression of female reproductive traits and determine its effects on copulatory behaviour. Populations of *C. maculatus* were raised at different temperatures (17^{o}C, 27^{o}C and 33^{o}C) in order to investigate the effects of developmental temperature on: 1) female reproductive trait anatomy, 2) copulatory behaviour and 3) the outcome of sperm competition.

Developmental temperature significantly affected female reproductive architecture. The bursa copulatrix of females grown at 17^{o}C tended to be longer and thinner than those grown at 27^{o}C or 33^{o}C. This was evident from linear and shape measurements. The bursa copulatrix is where females receive the spermatophore, therefore variation in the shape of this structure could influence how copulation proceeds in this species. The spermathecal duct length (a key feature of sperm-female coevolution) was shortest in those females grown at 17^{o}C, although not significantly so.

The developmental temperature experienced by both males and females affected the duration of copulation. Females from the 27^{o}C treatment group experienced shorter copulations than those from the 17^{o}C and 33^{o}C temperature groups, whilst males reared at 17^{o}C copulated for longer than males reared at the two warmer temperatures. Females reared at 17^{o}C were very reluctant to mate at all, with only 14% of 111 attempts resulting in copulation. This may be related to the bursa copulatrix of 17^{o}C females being abnormally shaped.

The effect of both male and female developmental temperature on the outcome of sperm competition was estimated using the genetic marker technique (colour polymorphisms). Both female and male developmental temperature had a significant effect on the outcome of sperm competition. P_2 was highest in those females reared at 33^{o}C whilst males from the 27^{o}C

treatment achieved higher P_2 values than males reared at either 17°C or 33°C. There was no effect of male nor female development temperature on P_1.

In conclusion, this research illustrates how a change in developmental temperature can affect the expression of primary reproductive traits in females and affect copulatory behaviour and the outcome of sperm competition. Given temperature can vary on a micro-climate scale as well as local and global scales, I argue that these results are important for those considering the evolution of primary reproductive traits, as variation in female reproductive architecture is likely to help maintain genetic variation in high-fitness male reproductive traits.

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Go raibh míle maith agaibh go léir!

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General Introduction

1.1 Literature Review

1.1.1 Primary reproductive traits

Primary reproductive traits have traditionally been considered as being directly associated with sexual reproduction; gametes, genitalia and seminal products (Ghiselin, 2010). By contrast secondary sexual traits (horns, antlers, bright plumage) are characteristics that appear at sexual maturity and are associated with out-reproducing one's conspecific rivals of the same sex (Ghiselin, 2010). Indeed, these two functions (gamete transfer and sexual competition) and their separate evolutionary mechanisms (natural and sexual selection respectively) have been integral to the definition of primary and secondary sexual characters (Darwin, 1871; Ghiselin, 2010). However, since Parker's pioneering work on sperm competition (Parker, 1970) it has become clear that many primary reproductive traits have evolved at least in part via sexual selection (Smith, 1984; Eberhard, 1996; Birkhead and Moller, 1998; Simmons, 2001; Leonard and Cordoba-Aguilar, 2010). Thus, distinguishing between primary and secondary reproductive traits has become increasingly difficult although Ghiselin (2010) suggests that possession of sperm-forming and egg-forming tissues equate to primary reproductive character differences. However, he also makes the point that if we are interested in relative differences between testes size, for instance, these differences may reflect differences in the intensity of sexual selection. The upshot of this is that it is difficult to define primary reproductive traits, although for the purpose of this dissertation I consider primary reproductive traits to be those associated with the transfer, delivery and utilization of gametes and thus to include the anatomical architecture of female and male reproductive tracts.

The large differences between species in the morphology of primary reproductive traits suggest these traits to have undergone rapid evolutionary divergence (Eberhard 1985; 1996). In an effort to explain the evolution of primary reproductive traits the 'Lock & Key' hypothesis was proposed (see Eberhard, 1985). This hypothesis suggests that female genitalia evolve in a manner that can be accessed only by males who have genitalia that have evolved to 'fit' with theirs in the manner of a lock and key. The theory suggests that female and male genitalia have evolved in an elaborate manner to prevent inter-species mating, thereby avoiding the production of costly hybrids (Eberhard, 1985). However, this hypothesis has met with frequent criticism (Eberhard, 1985; Arnqvist, 1998) and is generally considered unlikely to be the reason for species specific variation in reproductive traits (Arnqvist, 1998). For example, it is doubtful that a female would wait until genitalic contact had commenced before determining if the male

was of the desired species and thus natural section would most likely operate at a pre-copulatory level prior to genitalic contact (Eberhard; 1985, 2010). Furthermore, Arnqvist (1998) found male genitalia to be more divergent in polyandrous insect clades (Coleoptera, Diptera, Ephemeroptera, Lepidoptera) than in related monandrous clades. The lock and key hypothesis made no such prediction.

An alternative explanation for genitalic evolution is pleiotropy as proposed by Mayr in 1963 (see Eberhard, 1985; Simmons, 2014) who theorised that a gene could affect more than one trait. The reasoning behind this theory is that genitalia are held internally and as a result protected from the discerning eye of natural selection. Thus, they are free to accumulate pleiotropic alterations to structural morphology without affecting the survival of the individual. However, pleiotropy has been largely rejected as an explanation for the evolution of genitalia due to the fact that other internal structures are not affected in the same manner as genitalia. For example, digestive systems, circulatory systems and excretory systems are rarely used to distinguish between closely related species (i.e. have not undergone rapid evolutionary divergence) (Eberhard, 1985; Simmons, 2014).

1.1.2 Sexual selection

Up until the 1970s, the general consensus was that females were sexually monogamous and thus, sexual selection was considered a pre-copulatory event (Parker, 1970; Moller, 1998; Birkhead & Pizzari, 2002). However, the realisation that females (of many species from a wide variety of taxa) are not monogamous, meant sexual selection continued into the post-copulatory arena (Birkhead & Pizzari, 2002). Any trait that leads to a competitive advantage in the endeavour to fertilise is likely to be subjected to post-copulatory sexual selection via sperm competition and/or cryptic female choice (Møller, 1998; Birkhead & Pizzari, 2002; Eady, 2010).

Sperm competition is the contest between the sperm from two or more males to fertilise the female ova (Parker, 1970; Simmons, 2001; Birkhead & Pizzari, 2002; Snook, 2005; Pizzari & Parker, 2009). Sperm competition tends to select for two categories of male adaptation: sperm pre-emption and anti-sperm pre-emption (Parker 1970). Sperm pre-emption encompasses those male traits that dislodge, flush or numerically overwhelm the sperm of rival males already in the female reproductive tract, whilst anti-sperm pre-emption mechanisms encompass those male adaptations that protect self's sperm from being pre-empted (Simmons, 2001).

As a general rule when sperm competition occurs, males are more successful if their ejaculates contain more sperm (Parker, 1970; Birkhead & Pizzari, 2002). This is supported by the observation that species in which sperm competition is common generally have relatively larger testes, to populate ejaculates with a greater quantity of sperm, than species in which sperm competition is rare (Møller, 1998; Birkhead & Pizzari, 2002; Eberhard, 2010). Sperm competition also affects the form, function, morphology and quality of sperm. For example, Hunter and Birkhead (2002) looked at polyandrous and monandrous species from four different orders of insect (Coleoptera, Dictyoptera, Diptera & Hymenoptera) and found males belonging to polyandrous species tended to have lower sperm mortality than those of monandrous species. Furthermore, sperm length in the polyandrous bumblebee (*Bombus hypnorum*) is greater than that in their male counterparts of the monandrous species, *B. terrestris* and *B. lucorum* (Baer *et al*., 2003).

Cryptic female choice can be defined as the female exerting control over fertilisation through physical or chemical means to determine which males' sperm will fertilise her ova (Eberhard, 1996; Birkhead, 1998; Birkhead & Pizzari, 2002; Snook, 2005). It has been suggested that this mechanism, akin to Fisherian selection allows the female to produce male offspring with genitalia capable of effectively stimulating a female in the future to ensure reproductive success (Eberhard, 2010). Furthermore, cryptic female choice also acts on other male traits including sperm length and seminal products (Eberhard, 1996). For example, in the cricket family, Gryllidae, males transfer a spermatophore, which remains external to the female. The female can determine which male fertilises her ova by removing the spermatophore prior to the completion of sperm transfer (Vahed, 2015). Once inseminated, females can impose restrictions on the movement of sperm through morphological, physiological, chemical and behavioural means (Eberhard, 1996). Female field crickets (Gryllidae) exert control over the movement of sperm, preferentially allowing sperm that has come from an unrelated male, to move to the spermatheca and ultimately fertilise more of the female's ova (Vahed, 2015).

That females can strongly bias male success in sperm competition has been demonstrated by Wilson *et al*. (1997). They found that when the same two males were mated to several different females the outcome of sperm competition was not repeatable. However, when two males were mated to several different females that were genetically similar (full-siblings to one another but not related to the males), the outcome of sperm competition was highly repeatable. Furthermore, when Brown & Eady (2001) compared the outcome of sperm competition between geographically isolated populations of *Callosobruchus maculatus*, they found an effect of male and female population origin and an interaction between male and female population

origin on the extent of sperm precedence. Males were more successful when mated to a female from a population from the same geographical origin (see also Clark *et al.,* 1999 for further evidence of male-female interaction effects on the outcome of sperm competition).

With females having such an influence on the outcome of sperm competition, it follows that female reproductive architecture is likely to influence the evolution of male sperm traits. Several studies have shown a positive correlation between the length of sperm and the dimensions of the female sperm storage organs (Snook, 2005). For example, in featherwing beetles (*Bambara* spp.) sperm length correlates with the length of the ducts leading to the females' sperm storage organs. This points to a co-evolutionary process driving the evolution of male and female primary reproductive traits (Dybas & Dybas, 1981). Similar sperm-female tract correlations have been reported in stalk-eyed flies (Diopsidae) (Presgraves *et al*., 1999), *Drosophila* (Pitnick *et al*., 1999) and Bruchid beetles (Rugman-Jones & Eady, 2008). In *D. melanogaster*, Miller & Pitnick (2002) provide experimental evidence of sperm-female tract co-evolution. They artificially selected for long and short seminal receptacle lengths in replica lines of female *D. melanogaster*, whilst simultaneously selecting for long or short sperm in males in separate replica lines. During sperm competition trials staged between males with long and short sperm, females selected to have longer seminal receptacle length biased fertilization towards males with the longer sperm. Furthermore, in the lines in which females were selected to have long seminal receptacle lengths, males co-evolved to have longer sperm lengths (Miller & Pitnick 2002), suggesting female reproductive architecture influences the evolutionary trajectories of sperm length. Of particular relevance to the current study, Miller & Pitnick (2002) conclude that although it is now understood that sperm length evolution in *D. melanogaster* is driven by seminal receptacle length, it is not yet understood what drives the evolution of seminal receptacle length in this species. Therefore, a key question in our understanding of the evolution of sperm morphological diversity is: what drives the evolution of female reproductive architecture?

Female reproductive architecture could evolve via a mechanism akin to classic sexual selection (Simmons & Kotiaho, 2007; Eberhard, 2010) and/or via runaway sexual conflict (Rice, 1996). However, it is also possible that female reproductive architecture evolves in response to natural selection acting upon female life-history traits that require different patterns or processes of sperm storage. For example, in a comparative study of stylommatophoran gastropods Beese *et al*. (2009) found carrefour complexity (an area of the female reproductive tract associated with sperm storage) to be associated with reproductive strategy (semelparity versus iteroparity), reproductive mode (oviparity versus ovoviviparity) and habitat type. The results of Beese *et al*.

(2009) in combination with those of Miller and Pitnick (2002) set the scene for a post-copulatory equivalent of the sensory drive model of pre-copulatory trait evolution (Boughman, 2002). Essentially, natural selection selects for new sperm utilisation patterns that are associated with the evolution of novel life-history traits. These new sperm utilisation strategies are accompanied by the evolution of anatomical and physiological changes to the female reproductive tract (Beese *et al*., 2009; Yanagi & Tuda, 2012), which ultimately affect sperm function, placing selection on males to produce sperm that are adapted to the new environment. However, evidence in support of this mechanism is circumstantial and only a handful of studies have reported variation in female reproductive architecture in relation to environmental variables, and even here, the variation in female reproductive architecture can be evolved or plastic.

1.1.3 Phenotypic plasticity

Central to the argument above is understanding how male and female primary reproductive traits interact and how the female reproductive environment responds to environmental change. With regard to the female reproductive environment, it is clear that it can respond in both a plastic (Berger *et al*., 2011; Schäfer *et al*., 2013) and evolved manner (Beese *et al*., 2009; Yanagi & Tuda, 2012). Phenotypic plasticity is the ability of a single genotype to express more than one phenotype as a result of a change in the environment (Whitman & Agrawal, 2009). Differences in morphology, physiology, behaviour and life history traits can result from phenotypic plasticity (Whitman & Agrawal, 2009). These differences can be continuous (e.g. an increase in body size) or discrete (e.g. gregarious or solitary form of the locust) (Pfennig *et al*., 2010). One of the most important environmental stimuli, especially to poikilothermic animals is temperature. In mice exposed to testicular heat stress of 42^{o}C, sperm concentration, percentage of live sperm and sperm motility were significantly reduced (Pérez-Crespo *et al*., 2008). When these mice mated, there was no effect of heat treatment on pregnancy rate. However, there was a significant reduction in the average number of foetuses in comparison to the control group (Pérez-Crespo *et al.,* 2008). This was due to reduced implantation sites which Pérez-Crespo *et al*. (2008) report could be due to subfertility of the males or a reduction in embryo quality. Developmental temperature influences a wide-range of traits such as development time, body size, longevity, fecundity (Clarke, 2003; Angilletta, 2009; de Jong & van der Have, 2009) and importantly for the present study, male and female primary reproductive traits. It has been known for some time that developmental temperature determines sex in a number of species (Janzen, 1994; Angilletta, 2009). Thus, it is perhaps not surprising that developmental temperature also affects the phenotypic expression of sperm. Breckels & Neff

(2013) varied the temperature at which guppies (*Poecilia reticulata*) were reared from birth and found that higher temperatures resulted not only in shorter sperm but also slower moving sperm. Similarly, in the land snail (*Arianta arbustorum*), Minoretti *et al*. (2013) reported that varying development temperature affected sperm length; higher temperatures resulted in shorter sperm. In yellow dung flies (*Scathophaga stercoraria*), the opposite was found; males reared at higher temperatures produced longer sperm than those reared at cooler temperatures (Blackenhorn & Hellriegel, 2002), whilst in *C. maculatus*, males reared at intermediate temperatures produced the longest sperm (Vasudeva *et al*., 2014).

Female primary reproductive traits also respond plastically to changes in developmental temperature. The yellow dung fly (*Scathophaga stercoraria*), typically has three spermathecae (the 3S phenotype) although 4S phenotypes are evident at low frequency in natural populations (Berger *et al*., 2011). Berger *et al*. (2011) found the number of 4S phenotypes to increase with increasing developmental temperature. There was also a clinal variation with northern populations experiencing a greater occurrence of the 4s phenotype than the southern populations when reared under the same conditions in the laboratory. Given the significance of female reproductive architecture to both the outcome of sperm competition (Miller & Pitnick, 2002) and the coevolution of sperm length (Pitnick *et al*., 1999; Miller & Pitnick, 2002; Rugman-Jones & Eady, 2008), one of the key aims of the present study is to determine the extent to which developmental temperature affects the reproductive architecture of female *C. maculatus* beetles.

1.2 *Callosobruchus maculatus*

Callosobruchus maculatus is a beetle that originates from tropical/subtropical regions, such as Brazil, India and Niger (Beck & Blumer, 2014). Adult females lay up to 120 eggs on the seeds of legumes. The eggs are glued directly onto the seed surface and upon hatching the larvae burrow directly into the seed cotyledon where it feeds and excavates a chamber. Here the larva enters pupation and eventually emerges from the seed as a fully mature adult (Vasudeva *et al*., 2014). These beetles are a serious post-harvest pest of stored legumes. They have a short life-cycle (approximately 3 – 4 weeks) at 27^{o}C, which, in combination with the fact they readily colonise bean stores, makes them an ideal model species, as several generations per year can be easily maintained within a laboratory setting.

Copulation in *C. maculatus* follows two distinct phases: the start-to-kick phase and the kick-to-end phase. Phase 1 or the 'start-to-kick' phase describes the onset of genital coupling to a point

in copulation (typically a few minutes after genital coupling) in which the female begins to kick at the male with her hind legs (Eady 1994b). Much of the ejaculate is transferred during this stage (Vasudeva, 2014). Phase 2 or the 'kick-to-end' phase is the time from the onset of female kicking behaviour to the genital de-coupling of the pair (Eady, 1994b, Edvardsson & Canal, 2006; Vasudeva, 2014).

The parental stock population for this research originated from Niamey, Niger and had been kept on black-eyed beans (*Vigna ungliculata*) for more than 100 generations at the University of Lincoln. There are two distinct colour morphs of *C. maculatus* maintained at the University of Lincoln. The wild-type morph (WT) are mainly tan in cuticular colour, with patches of black on the elytra (Eady, 1991). The black-type morph (BT) were cultured by Eady from a black mutation (Eady, 2015 *pers. comm*.) and are completely black, including the legs, apart from small patches of white pubescence on the pronotum, elytra and pygidium. When individuals from the black-type morph are crossed, all offspring are phenotypically black. When WT individuals are crossed, all offspring are WT in appearance. By contrast, when BT and WT are crossed, the resulting offspring are predominantly black with tan fore- and mid-legs and tan segments on the antennae (Eady, 1991). The important point here is that the two colour morphs can be used to estimate paternity in sperm competition experiments. The outcome of sperm competition is often given as a species-specific P_2-value where P_2 is the proportion of offspring resulting from the sperm of the last male in a sequence to mate with a female (Eady, 1991; Simmons, 2001). To determine the success of an individual during sperm competition, controlled mating trials tend to be performed in which a female is mated to two males with the result recorded as P_2 (Birkhead & Pizzari, 2002; Eady, 2010). However, such mating trials tend to not take into account the role of the female which is where sperm competition generally occurs (Snook, 2005; Rugman-Jones & Eady, 2008).

1.2.1 Effect of temperature on *C. maculatus*

Recently Vasudeva (2014) and Vasudeva *et al*. (2014) reared *C. maculatus* at varying temperatures (17°C, 25°C, 27°C and 33°C) and conducted several experiments to determine the effect of developmental temperature on a range of traits, including the primary reproductive traits of males. *C. maculatus* grown at 17°C took considerably longer to develop than those reared at the other temperatures – 87 days as opposed to the average of 25 days (Vasudeva, 2014). Upon eclosion, adults reared at 17°C were significantly larger than adults grown at the higher temperatures (Vasudeva, 2014; Vasudeva *et al*., 2014). Males reared at 17°C had the largest absolute testes size, but due to them being of greater body size, relative testes size was

largely unaffected by developmental temperature (Vasudeva *et al*., 2014). Developmental temperature had an effect on sperm length with the longest sperm being produced by males reared at 27°C (Vasudeva, 2014; Vasudeva *et al*., 2014). Furthermore, copulation duration was affected by developmental temperature with both the start-to-kick phase and overall copulation duration taking longer for males reared at 17°C (Vasudeva, 2014). Interestingly males reared at 17°C also performed least well during sperm competition experiments while males reared at 27°C were most successful at P_2 (Vasudeva *et al*., 2014). This is not surprising, considering that males reared at 17°C transferred the fewest sperm in comparison to all other treatment group (Vasudeva, 2014; Vasudeva *et al*., 2014). Vasudeva (2014) also found that females previously reared at 17°C laid fewer eggs than females from the other treatment groups, but he did not analyse other female reproductive characteristics.

Plasticity in female reproductive traits could prove important in understanding the evolution of primary reproductive traits because there is good evidence that male and female reproductive traits co-evolve (Dybas and Dybas, 1981; Pitnick *et al*., 1999; Miller & Pitnick, 2002; Rugman-Jones & Eady, 2008) and that this co-evolutionary process may at least in part be driven by variation in female reproductive architecture (Miller & Pitnick, 2002; Simmons & Kotiaho, 2007). Therefore, this project primarily sets out to determine the effect of developmental temperature on female reproductive architecture, copulatory behaviour and the outcome of sperm competition.

1.3 Aims and Hypotheses

The aims of this study were to determine the effect of developmental temperature on:

- phenotypic plasticity in female reproductive architecture.
- the duration of copulation.
- the outcome of sperm competition.

Considering the information presented above the following hypotheses are postulated.

- Given there is evidence, albeit limited, of temperature driven plasticity in female reproductive architecture in *Scathophaga* stercoraria (Berger *et al*., 2011; Schafer *et al*., 2013) it was predicted that developmental temperature would affect the reproductive architecture of female *C. maculatus*.
- In addition, because females are active participants in the copulatory behaviour of this species (Eady and Brown, MS), it is predicted that developmental temperature experienced by females would affect the duration of copulation.

- Finally, because female reproductive architecture appears to be closely linked to male success in sperm competition (Miller & Pitnick, 2002) it was predicted that developmental temperature experienced by females would affect the outcome of sperm competition.

1.4 General Methodology

The effect of temperature on the development of *Callosobruchus maculatus* from egg to adult was investigated by rearing beetle larvae at three different temperatures (17^{o}C, 27^{o}C and 33^{o}C). To initiate the cultures, newly eclosed adult beetles were placed on approximately 200g of moth beans (*Vigna aconitifolia*) to lay eggs for 24 hours at 27^{o}C. Moth beans were used because on average only one adult beetle emerges per bean, thus allowing greater control over resource competition and in obtaining virgin adults of known age (Vasudeva, 2014). Beans with eggs were separated into three groups and placed in incubators at 17^{o}C, 27^{o}C and 33^{o}C under continuous light. Panasonic Cooled Incubator MIR-154 Series incubators were used to culture the beetles at the required temperature. Following Vasudeva (2014) approximately one to two days prior to eclosion, individual moth beans with eclosing larvae were transferred into separate cells of 5x5 repli-dishes, fitted with glass lids in order to isolate virgin adults of known age following emergence.

Chapter 2
The effect of temperature on the development of female reproductive traits in *Callosobruchus maculatus*

2.1 Introduction

The use of primary reproductive traits to differentiate between species indicates that these characters have undergone rapid and divergent evolutionary change (Eberhard, 1985; Leonard & Cordoba-Aguilar, 2010). It also suggests that these traits are well canalised, meaning the genotype is able to produce the same phenotype in the face of environmental stress (Waddington, 1942). However, regardless of the evidence for canalisation in genitalia (Eberhard, 2008) examples exist which demonstrate environmental factors to influence the development of male and female primary reproductive traits (Houck & Verrell, 2010).

When varying the diet of the small hive beetle (*Aethina tumida*), de Guzman *et al*. (2015) describe how a lack of pollen in the diet resulted in ovaries not being activated, leading to reproductive failure in these beetles. Marshall (2007) found that the spermathecal duct length of three species of cricket belonging to the *Allonemobius socius* complex, varied in relation to species, population and *Wolbachia* infection status. Infection with *Wolbachia* resulted in an increase or decrease in the length of the spermathecal duct dependent upon the population of cricket and/or the strain of *Wolbachia*. Berger *et al.* (2011) reared yellow dung flies (*Scatophaga stercoraria*) from different latitudes at varying temperatures and found that northern populations grew faster than southern populations at the same environmental temperature of 12^{o}C. In addition, individuals reared at lower temperatures were more likely to have an increased number of spermatheca (Berger *et al.*, 2011).

Sperm length has also been shown to differ in relation to a variety of environmental factors. When virgin male cockroaches (*Nauphoetaa cinerea*) were mated a second time immediately after their first mating, the sperm transferred during the second mating were significantly shorter than those transferred during the first mating (Harris *et al*., 2007). When kept at varying temperatures from birth, guppies (*Poecilia reticulata*) not only produced significantly shorter sperm as holding temperature increased but the sperm produced also moved more slowly than sperm from males held at control and lower temperatures considered the norm for this species (Breckels & Neff, 2013). More recently, Vasudeva (2014) discovered that manipulating rearing temperature of the beetle *Callosobruchus maculatus* had an effect on the male reproductive

characters. Individuals reared at temperatures that were lower or higher than the optimal developmental temperature both had shorter sperm.

The fact that male and female reproductive traits express plasticity in relation to environmental conditions suggests that environmental conditions experienced during development might affect the outcome of sperm competition and influence the nature of sexual selection experienced by males and females. Eberhard (1996) has argued the female reproductive environment sets the rules by which sperm competition is played out. Indeed, there is good evidence that male and female primary reproductive traits co-evolve. For example, the comparative method has revealed sperm length to co-vary with dimensions of the female reproductive tract in a number of taxa including featherwing beetles (Dybas & Dybas 1981), *Drosophila* (Pitnick *et al*., 1999) and bruchid beetles (Rugman-Jones & Eady 2008). Such patterns of co-evolution appear to be driven by the interactions between sperm and female reproductive architecture. For example, Miller & Pitnick (2002) artificially selected for long or short seminal receptacle (SR) lengths in female *Drosophila melanogaster* and at the same time, but in different populations, long and short sperm lengths in males. After several generations of selection, Miller & Pitnick (2002) competed long versus short sperm against each other within females that had either long or short SR lengths. The long sperm outcompeted the short sperm but only when the competition took place within females that had long SR lengths, clearly demonstrating that the female reproductive environment affects the outcome of sperm competition. Of interest, Miller & Pitnick (2002) also reported a co-evolutionary response of sperm length to selection on female seminal receptacle length: males evolved longer sperm in those populations in which females were selected to have longer seminal receptacle lengths.

Due to the coevolution of reproductive traits in other species, it is fair to assume that not only the size of the reproductive tract may change, but also the shape of the tract. To investigate any changes in shape, a different method of analysis is required and in recent years, geometric morphometrics has become a standard method. Geometric morphometrics (GMM) is the analysis of shape by using coordinates to ascertain any change in shape. Size is removed from the statistical analysis and the coordinate data are analysed instead (Polly, 2012; Zelditch *et al*., 2012). GMM allows researchers to compliment simple measurements, such as length and width, by being able to focus on both group and individual differences (Friedrich, 2013). The use of GMM provides a more in-depth view of the interaction between phenotype, genotype and the environment (Jensen, 2003).

GMM has been used in a variety of situations. These include: identifying hybrid tree species (Viscosi & Cardini, 2011); quantifying the effect of environmental variables on otoliths in fish (Bolles & Begg, 2000); distinguishing between morphotypes in whiting (Keating *et al*., 2014); revealing correlations between pelvis shape and egg shape (Anten-Houston, 2015); and comparing egg shape of extant and Mesozoic birds (Deeming & Ruta, 2014). It has also been used in the analysis of primary reproductive traits in invertebrates (Arnqvist, 1998; Simmons & Kotiaho, 2007).

Here the aim is to use GMM to study how developmental temperature affects female reproductive architecture in *C. maculatus*. In *C. maculatus*, the bursa copulatrix receives the spermatophore from the male. After a short time, sperm begin to move from the bursa copulatrix to the spermatheca via the spermathecal duct (Eady, 1995). There is a circular valve-like structure (bursal valve) that is present on the bursa copulatrix (Mukerji & Hakim Bhuya, 1937) but its function is currently unknown. However, during preliminary investigation of the reproductive anatomy it was observed that the bursal valve appeared to be different across the experimental groups. Here the size and shape of the bursa copulatrix and the bursal valve were measured. The spermathecal duct is used as a conduit for the sperm to travel from the bursa copulatrix to the spermatheca. A comparative study by Rugman-Jones & Eady (2008) revealed evidence of sperm length co-evolution with the length of this duct. Thus, here the length of the spermathecal duct was measured.

2.2 Materials and Methodology

2.2.1 Preparation of the reproductive tract

To determine the effect of developmental temperature on female reproductive morphology, virgin females were collected 0-24 hours post-eclosion from all three temperature groups (17^{o}C, 27^{o}C & 33^{o}C) and were stored in a -20^{o}C freezer prior to analysis. Twenty females from each temperature group were selected at random for analysis. The reproductive tract was removed through the posterior end of the female using forceps. At this stage in the process the reproductive tract is covered in connective and fatty tissue, which impedes a clear view of the spermathecal duct and the spermatheca. To remove the connective and fatty tissue from the reproductive tract, a 1.13M solution of lactic acid was used.

This 10% solution was prepared using distilled water and a 90% lactic acid (2-Hydroxypropanoic acid) 11.3M solution. Each specimen was placed in an Eppendorf tube with 0.5ml of 1.13M lactic acid and placed on a heat block set at 90°C for 2.5 to 3 hours. The lactic acid was then removed from the tube with a pipette and the specimen rinsed 2-3 times in insect saline. The specimen was then stored in an Eppendorf tube containing 0.5ml of insect saline until examination.

2.2.2 Dimensions and shape of the female reproductive anatomy

The elytra were removed using forceps from each female used in this section of the study. Elytra length was measured by photographing the ventral side of each elytra and using the Straight Line tool in ImageJ (Rasband, 2015) to measure the longest length of the elytra. This is a good proxy for female body size (Wilson & Hill, 1989; Eady, 1994a; Timms, 1998). The female reproductive traits measured were: the length, width and shape of the bursa copulatrix (Figure 2.1) and the bursal valve and the length of the spermathecal duct (Figure 2.2).

An Olympus dissecting microscope [SZX12] connected to an image analysis work station (Moticam 3) was used in the dissection of the female reproductive tract and for the capture of images for subsequent analysis. Prior to being cleaned in the lactic acid solution the female reproductive tracts for each individual were photographed in the left lateral plane to allow measurement and shape analysis of the bursa copulatrix and the bursal valve (Figure 2.1). The straight line tool on ImageJ was then used to measure the length of the bursa copulatrix from the distal end of the bursal valve to the end of the bursa copulatrix (Figure 2.1a). The width was measured across the breadth of the bursa copulatrix just under the bursal valve (Figure 2.1b). The length of the bursal valve was measured across its longest length (Figure 2.1c) and the width was measured similarly across the widest points (Figure 2.1d).

The same set of images were also used to apply shape analysis to the bursa copulatrix and the bursal valve (Figure 2.3a & 2.3b). The image analysis program tpsDig 2.17 (Rohlf, 2005) was used to digitise the shape by applying equally spaced landmarks around the outline of the bursa copulatrix until the curve of the bursa copulatrix tapered off on both sides of the structure at the line parallel to the beginning of the bursal valve (Figure 2.3a). The same method was applied to the bursal valve but around the entire outline of this structure (Figure 2.3b).

Images of the spermathecal duct taken after the lactic acid treatment were used in ImageJ to measure spermathecal duct length from where it left the bursa copulatrix to the point where it visibly met the spermatheca (Figure 2.2). The duct length was measured using ImageJ's Segmented Line tool because several of the spermathecal ducts were curved.

2.2.3 Data Analysis

2.2.3.1 Dimensions analysis

The data were subjected to Levene's test to ensure parametric tests could be applied. The results showed all data were normally distributed and homogenous variance. IBM SPSS 22 was used to perform a one-way analysis of variance (ANOVA) to analyse differences among the temperature treatments for elytra length. An analysis of covariance (ANCOVA) determined the effect of temperature whilst controlling for body size measured as elytra length on the linear dimensions of the bursa copulatrix, the bursal valve and the spermathecal duct length. If the interaction term was non-significant ($p > 0.05$) then it was removed from the model and the significance of the factor and covariate tested. Post-hoc Tukey tests were carried out where applicable.

2.2.3.2 Shape analysis

Digital coordinates were imported into MorphoJ v.1.60d (Klingenberg, 2011). Following a Procrustes transformation and generation of a covariance matrix, Principal Components Analysis (Procrustes PCA) was performed to determine the pattern of variation in shape. PAST v2.17c (Hammer, 2001) was used to perform a one-way Non-Parametric Multivariate Analysis of Variance (NPMANOVA) analysis on each axis of Principal Component (PC) scores to investigate differences among the temperature groups. Scree plots produced using MorphoJ illustrated the movement of landmarks in each of the principal components.

2.3 Results

2.3.1 Elytra length/Body size

Females grown at 17°C had the longest elytra whilst those grown at 27°C had the shortest (Figure 2.4) and elytra length differed significantly between the temperature groups (One-way ANOVA: $F_{2,56} = 17.955$, $p < 0.0001$). A Tukey post-hoc test revealed that females from the 17°C temperature group had significantly longer elytra than both the 27°C ($p < 0.0001$) and 33°C ($p = 0.002$) females. Females from the 27°C group had shorter elytra than the 33°C females, although post-hoc analysis revealed this difference only approached significance ($p = 0.072$).

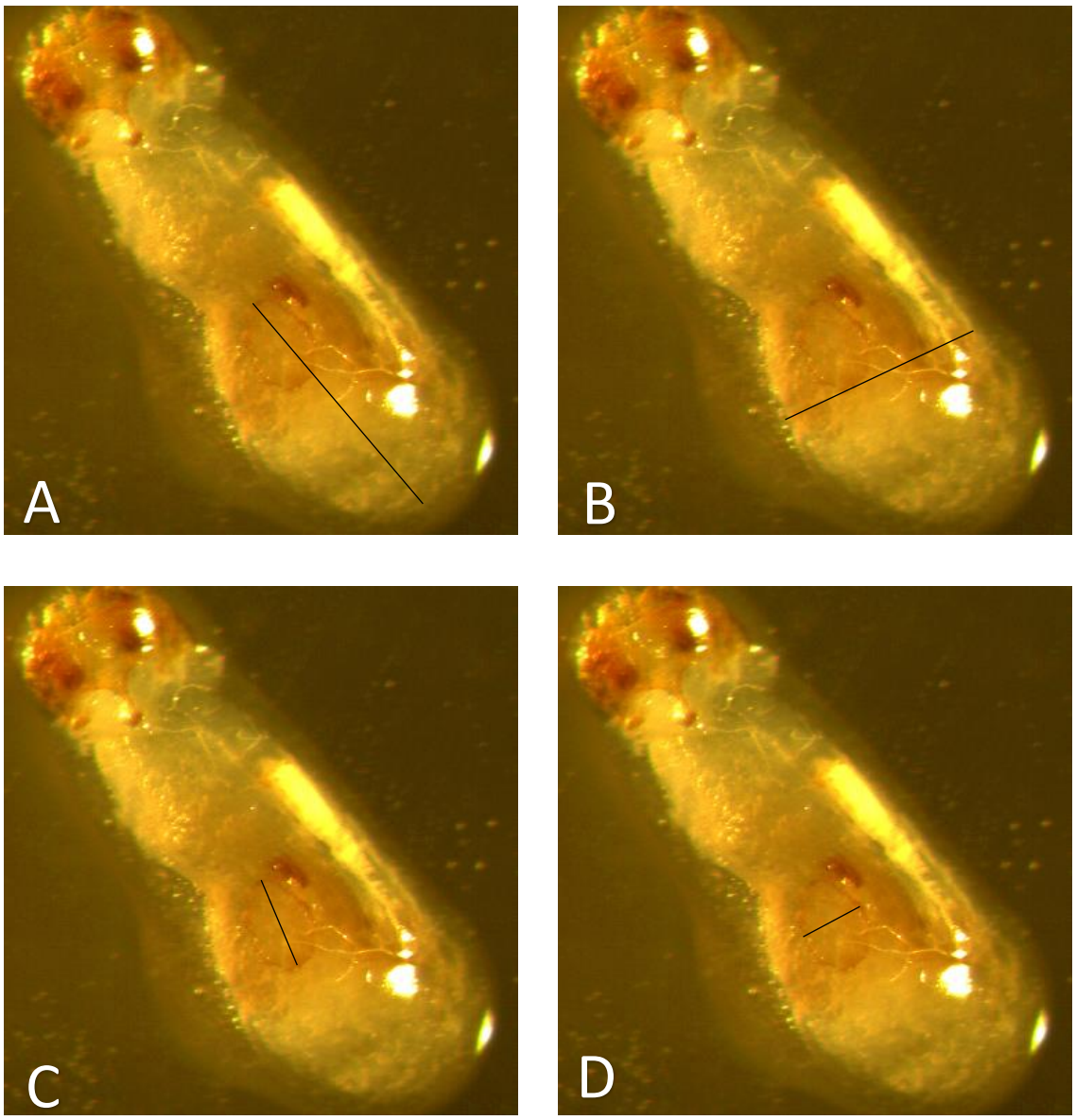


Figure 2.1. Example of (A) female reproductive tract and the line where measurements were taken for (A) length of bursa copulatrix, (B) width of bursa copulatrix, (C) length of bursal valve and (D) width of bursal valve.

Figure 2.2. An example how ImageJ was used to determine length of spermathecal duct.

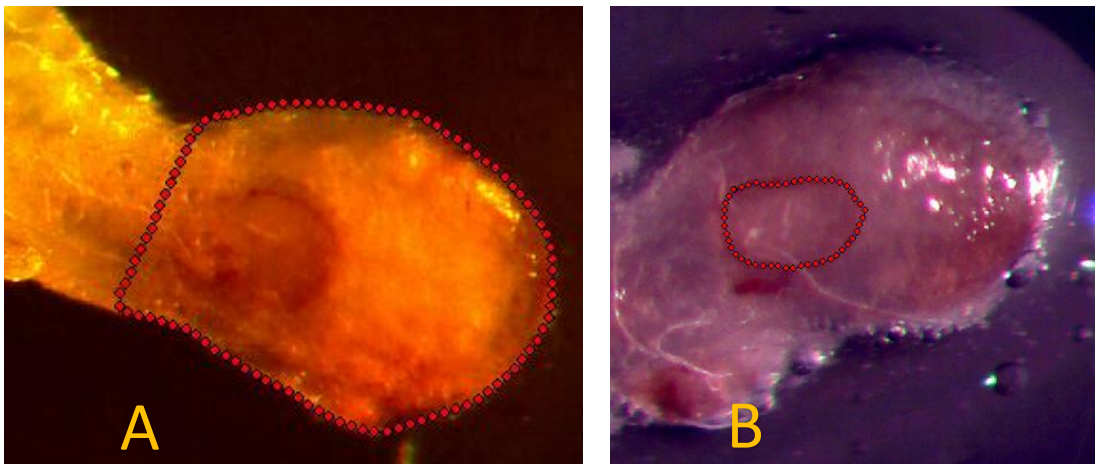


Figure 2.3. Examples of (A) 150 homologous landmarks applied to the bursa copulatrix and (B) the 50 homologous landmarks applied to bursal valve.

2.3.2 Length and width of the bursa copulatrix and the bursal valve

As developmental temperature increased the length of the bursa copulatrix tended to decrease. (Figure 2.5). ANCOVA revealed no significant interaction between developmental temperature and elytra length on the length of the bursa copulatrix ($F_{2, 53} = 1.465$, $p = 0.24$). Without the interaction term the model revealed a significant effect of temperature ($F_{2, 55} = 8.965$, $p < 0.0001$) but no effect of elytra length ($F_{1, 55} = 0.117$, $p = 0.733$) on bursa copulatrix length. Post-hoc tests following the subsequent one-way ANOVA revealed 33°C females to have significantly shorter bursas than those incubated at 17°C or 27°C (Figure 2.5).

The widest bursas were found at a developmental temperature of 27°C (Figure 2.5). ANCOVA revealed no significant interaction between developmental temperature and elytra length on the width of the bursa copulatrix ($F_{2, 53} = 0.147$, $p = 0.86$). Removal of the interaction term from the model indicated a significant effect of temperature ($F_{2, 55} = 9.916$, $p < 0.0001$) but no effect of elytra length ($F_{1, 55} = 0.313$, $p = 0.578$). Post-hoc tests following a one-way ANOVA of the effect of temperature showed that 27°C females had significantly wider bursas than those incubated at 17°C or 33°C (Figure 2.5).

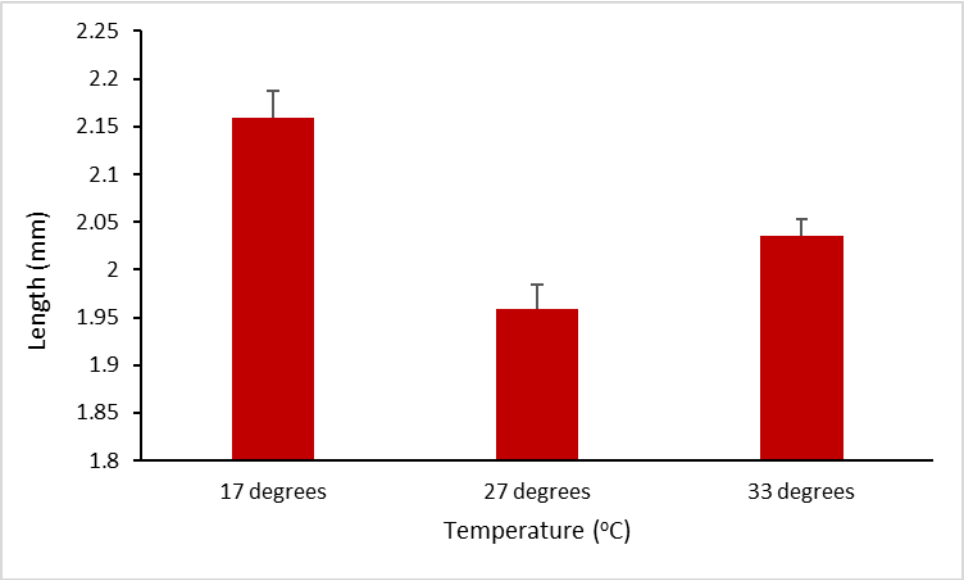


Figure 2.4 Mean (+SE) elytra length (mm) of female *C. maculatus* cultured at three different temperatures; for 17°C – n = 20, 27°C – n = 20 and 33°C – n = 19.

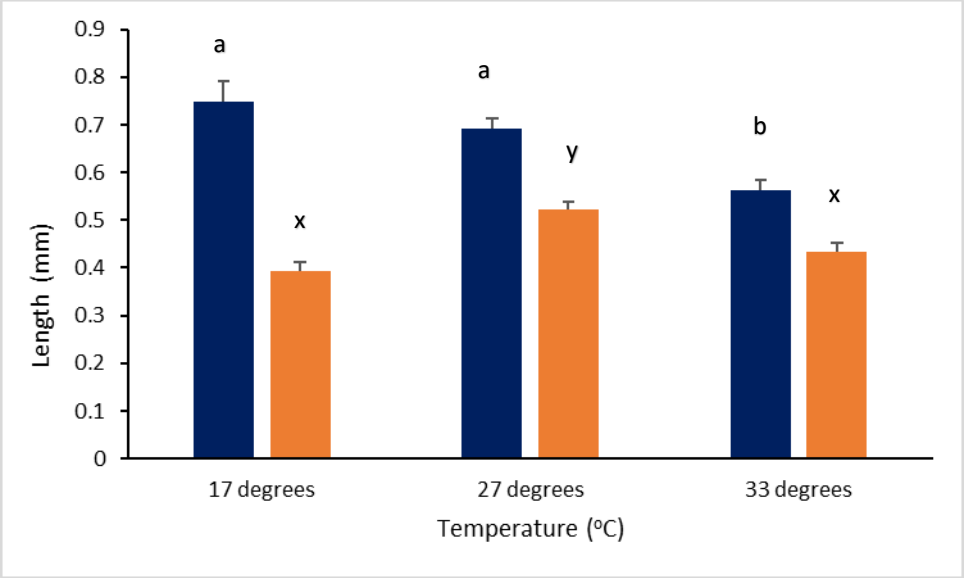


Figure 2.5 Mean (+SE) length (mm, blue columns) and width (mm, orange columns) of bursa copulatrix in female *C. maculatus* cultured at three different temperatures; n = 20 for each treatment group. Different letters above bars indicate significant differences between treatments as revealed by post-hoc tukey test ($p < 0.05$) as calculated from a one-way ANOVA.

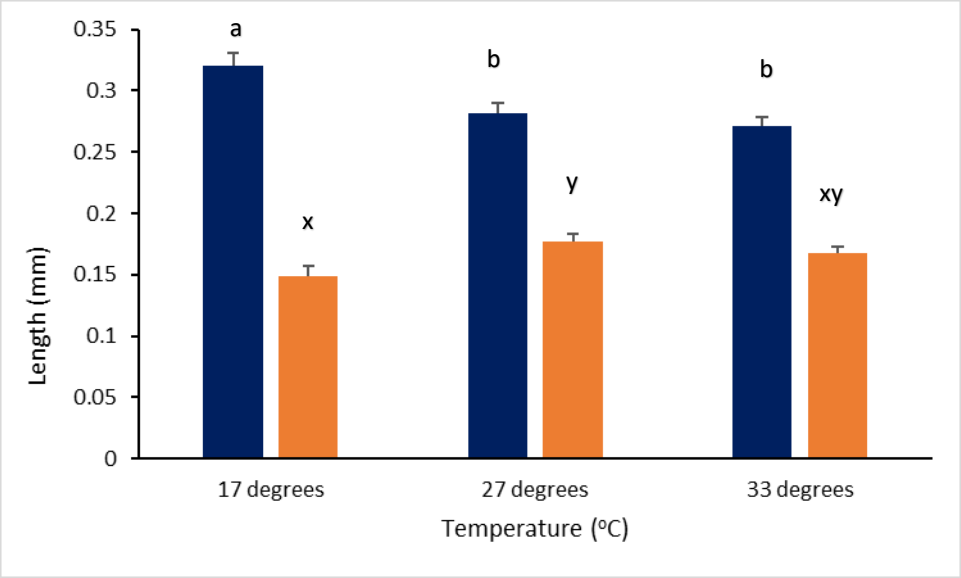


Figure 2.6 Mean (+SE) length (mm, blue columns) and width (mm, orange columns) of the bursal valve in female *C. maculatus* cultured at three different temperatures; n = 20 for each treatment group. Different letters above bars indicate significant differences between treatments as revealed by post-hoc tukey test ($p < 0.05$) as calculated from a one-way ANOVA.

The dimensions of the bursal valve followed a similar pattern to those found for the bursa copulatrix where bursal valve length increased with decreasing temperature whilst bursal valve width was smallest in those females that developed at 17°C (Figure 2.6). ANCOVA revealed no significant interaction between developmental temperature and elytra length on the length of the bursal valve ($F_{2, 53} = 2.057$, $p = 0.138$) but there was a significant effect of temperature ($F_{2, 55} = 5.978$, $p = 0.004$) on bursal valve length after the interaction was removed. Post-hoc tests following a one-way ANOVA revealed 17°C females to have longer bursal valves than those incubated at 27°C or 33°C (Figure 2.6).

There was no effect of elytra length ($F_{1, 55} = 0.029$, $p = 0.866$) on bursal valve length. For the width of the bursal valve there was no significant interaction between developmental temperature and elytra length; ANCOVA, ($F_{2, 53} = 0.019$, $p = 0.981$). The simpler model revealed a significant effect of temperature ($F_{2,55} = 3.785$, $p = 0.029$) but no effect of elytra length ($F_{1,55} = 0.68$, $p = 0.413$) on bursal valve width (Figure 2.6). Post-hoc tests following a one-way ANOVA revealed 17°C females to have significantly narrower bursal valves than those incubated at 27°C (Figure 2.6).

2.3.3 Spermathecal duct length

There were less spermathecal ducts available for analysis from females reared at 33°C (n = 14) than in the other two temperature groups (n=19 for both groups). ANCOVA revealed no significant interaction between developmental temperature and elytra length on the length of the spermathecal duct ($F_{2, 45}$ = 2.222, p = 0.12). The simpler model revealed no effect of either temperature ($F_{2,47}$ = 0.866, p = 0.427) or elytra length ($F_{1, 47}$ = 0.001, p = 0.97) on spermathecal duct length (Figure 2.7).

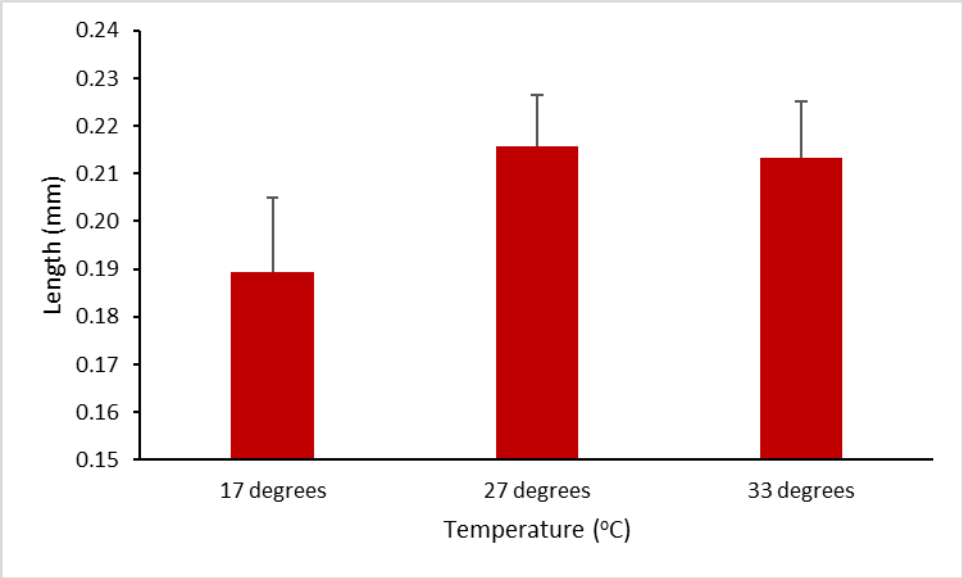


Figure 2.7 Mean (+SE) spermathecal duct length (mm) of female *C. maculatus* cultured at three different temperatures; for 17°C – n = 19, 27°C – n = 19 and 33°C – n = 14.

2.3.4 Shape of female reproductive anatomy

2.3.4.1 Bursa copulatrix

Approximately 75% of the variance observed in the shape of the bursa copulatrix was explained by Principal Components 1 and 2 (PC1 & PC2 in Figure 2.8). The scree plots (Figure 2.8) show that PC1 of the bursa copulatrix is oval and long (e.g. see image F116 in Figure 2.9) but the variation in the structure is such that the stucture becomes shorter and wider (e.g. see image F315; Figure 2.9). PC2 describes the bursa coplatrix being stretched (or shortened) on the diagonal and so becoming more rhomboid in shape. For example, image F117 (Figure 2.9) shows a bursa that is long and wide while the specimen in image F311 is long and narrowing, demonstrating this variation (Figure 2.9).

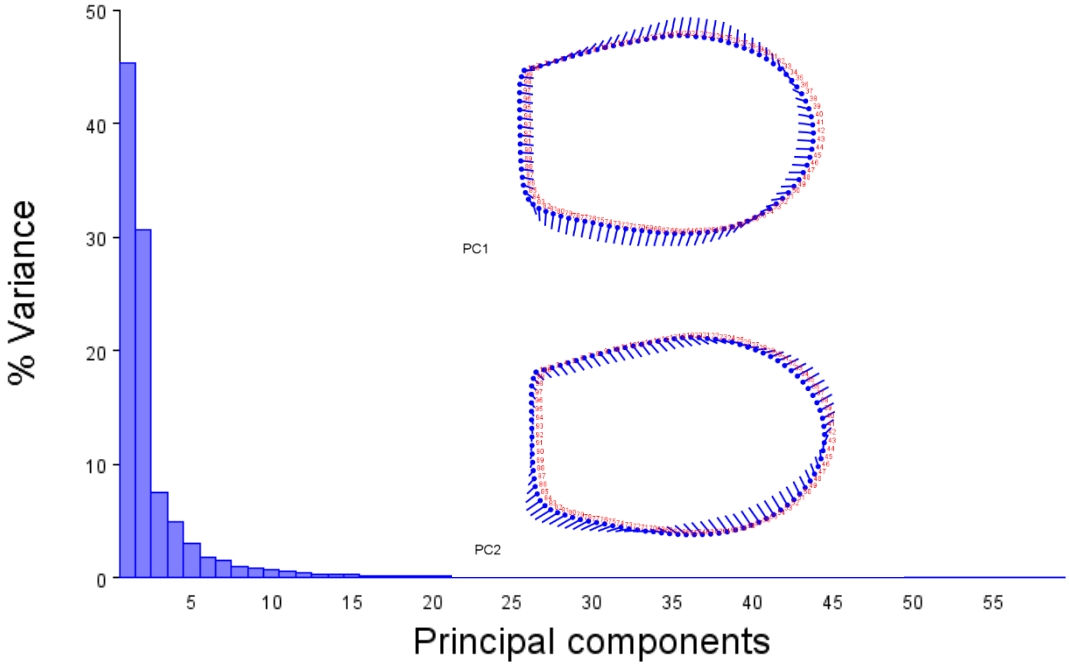
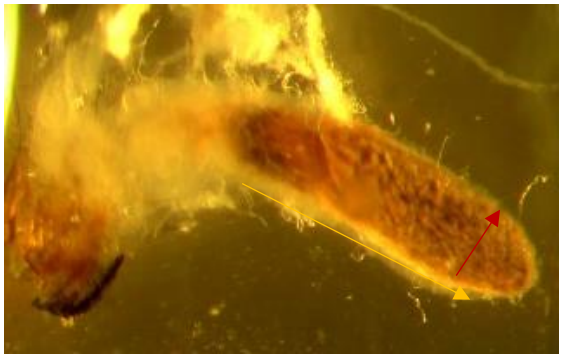
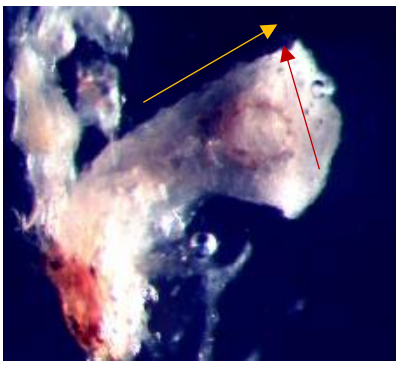


Figure 2.8 Variance (%) in shape data explained by the principal components making up the Principal Components Analysis of bursa copulatrix shape for females from the three treatment groups (17°, 27° and 33°C). Also shown are the scree plots of PC1 and PC2 indicating the direction of shape variance. The right-hand side of the scree plot is the distal end of the female reproductive tract.

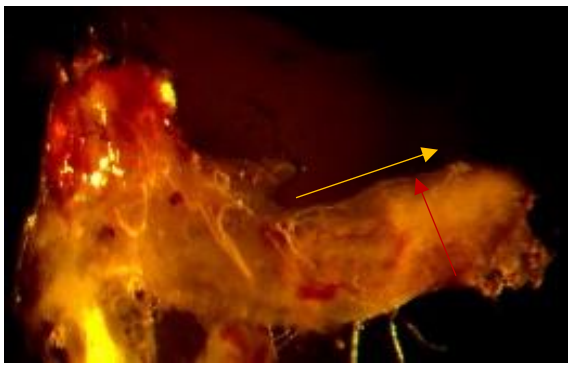
F116



F315



F117



F311

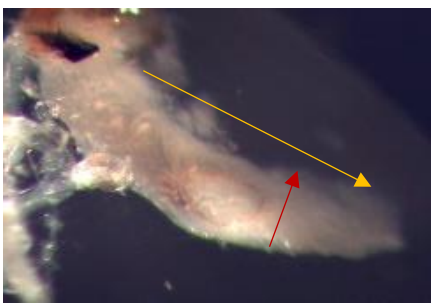


Figure 2.9 Images of the bursa copulatrix representing the outlying individuals as identified by the morphospace plot for both PC1 and PC2. Length denoted by the yellow arrow and width denoted by the red arrow.

The scatterplot of PC1 and PC2 suggested that the shape of the bursa copulatrix in the 27°C degree treatment group to be less variable in shape than both the 17°C and the 33°C females (Figure 2.10). However, this was only the case for the PC2 axis (Homogeneity of Variance test: L = 3.641, df = 2, 57, p = 0.032). Only six individuals from the 17°C treatment group sat within the space created by the 27°C group, whereas just over half the 33°C treatment group occupied this space. On PC1, increasingly elongated (respectively, wider) bursas are associated with increasingly negative (respectively, positive) scores. On PC2, increasingly thick (respectively, thinner) bursas are associated with increasingly negative (respectively, positive) scores. An NPMANOVA showed that this distribution was highly significant ($F_{2, 57}$ = 9.53, p = 0.0001). Furthermore, pairwise comparisons indicated that the 17°C and 27°C animals were significantly different from each other (p = 0.0001), as were the 17°C and 33°C animals (p = 0.0001). By contrast, the 27°C and 33°C animals were not significantly different (p = 0.754).

2.3.4.2. Bursal valve

Approximately 90% of the variance observed in the shape of the bursal valve is explained by PC1 and PC2 (Figure 2.11). For PC1 the bursal valve is on average an oval (see image F108; Figure 2.12). However, variation in the PC1 points to shape variation that is reflected by the oval being longer on the diagonal and narrower on the opposing diagonal (i.e. slightly elongated – see image F311 which is long and wide in Figure 2.12). On average PC2 showed a valve shape that was elongated but becoming a little more wide at the proximal end of the valve. Image F106 (Figure 2.12) portrays a long bursal valve that widens as it lengthens while F112 is an example of a long bursal valve that is consistent in its width throughout (Figure 2.12). Both these examples and the example F108 are from the 17°C treatment group, illustrating how variable the bursal valve shape is within this treatment group.

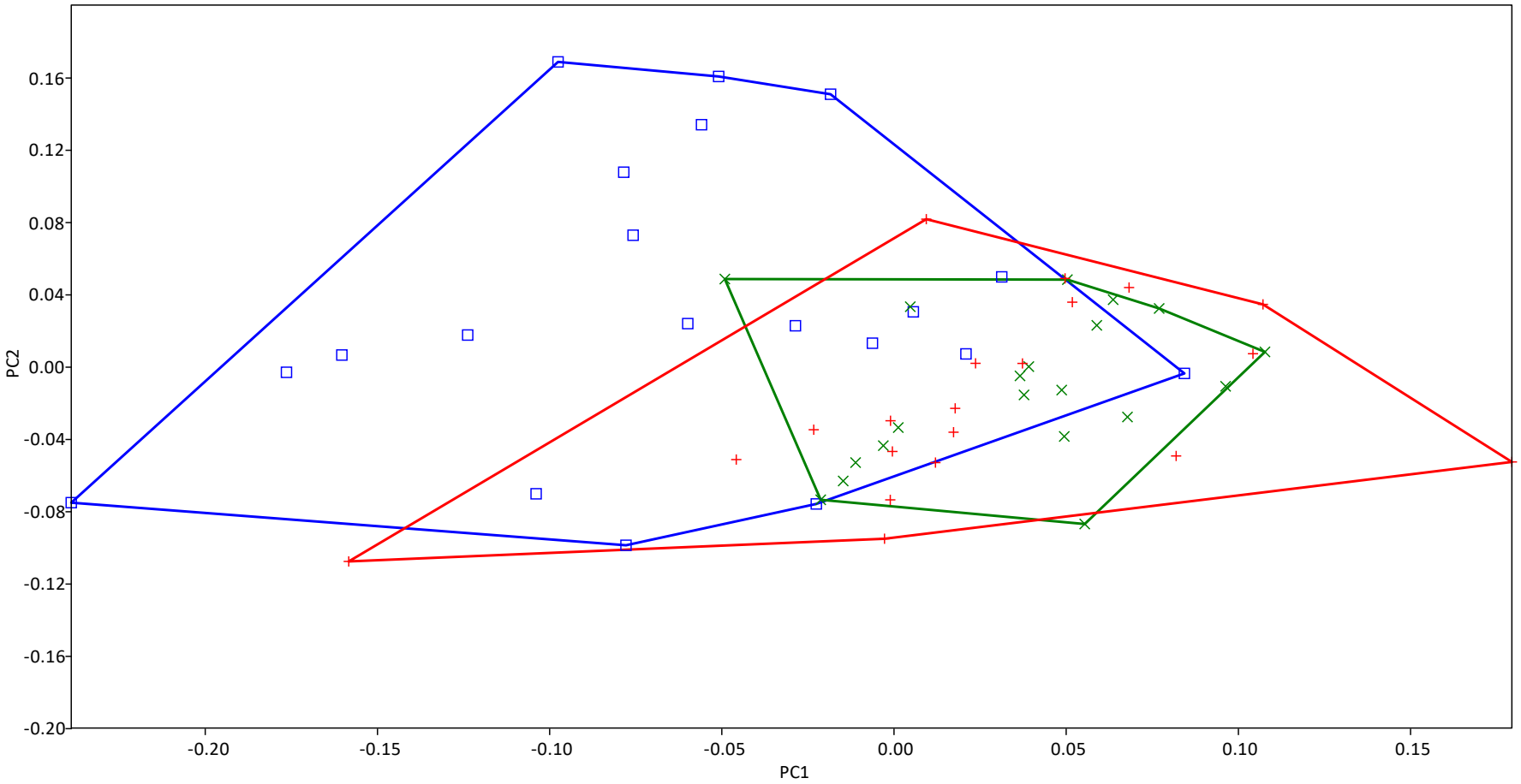


Figure 2.10 Convex hulls plot based on PC scores generated from the analysis of 60 outlines of bursa copulatrix. Colours and lines indicate the three temperature groups in the set. (Blue = 17°C, Green = 27°C, and Red = 33°C).

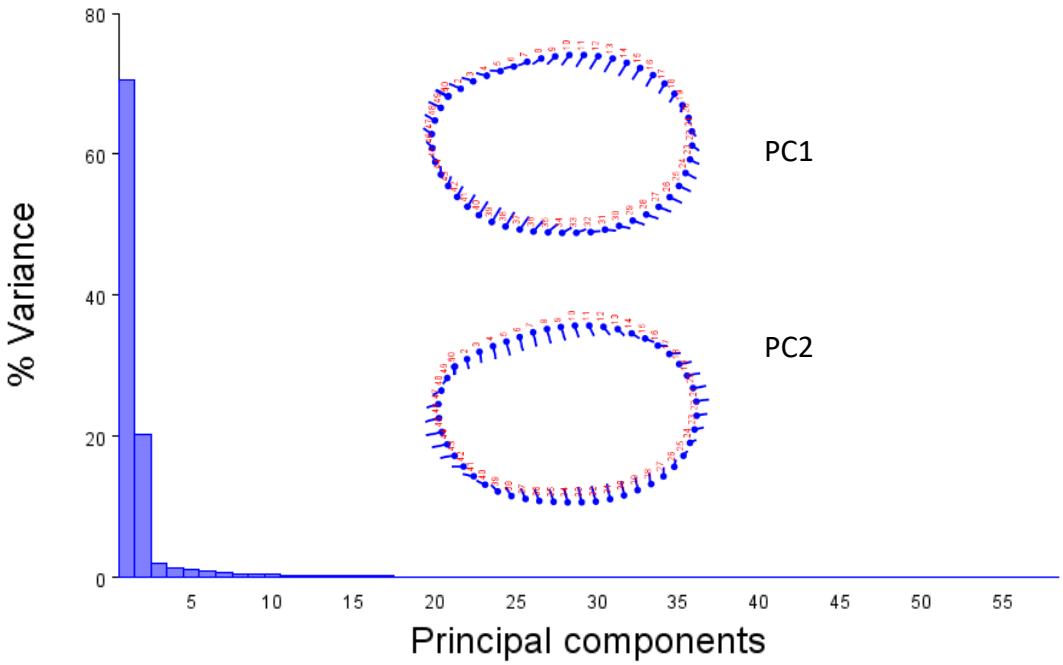


Figure 2.11 Variance (%) in shape data explained by the principal components making up the Principal Components Analysis of bursal valve shape for females from the three treatment groups (17°, 27°and 33°C). Also shown are the scree plots of PC1 and PC2 indicating the direction of shape variance.

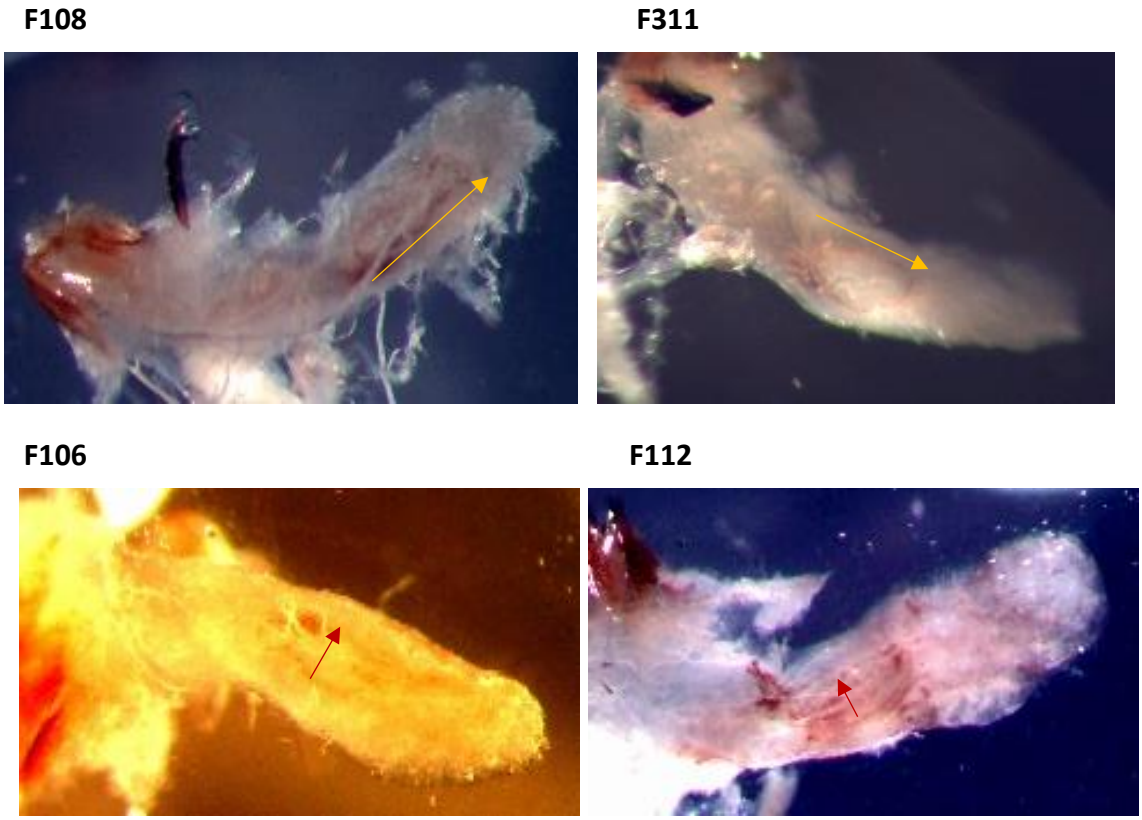


Figure 2.12 Images of the bursal valve representing the outlying individuals as identified by the morphospace plot for both PC1 and PC2. Length denoted by the yellow arrow and width denoted by the red arrows.

The 27°C treatment group were less variable in regards to bursal valve shape (Figure 2.13) but this was only significant for the PC1 axis (Homogeneity of Variance test: L = 18.628, df = 2, 57, p < 0.0001). As with the scatterplot for the bursal valve, very few of the 17°C treatment group (n=2) occupied the space created by the 27°C group while half of the 33°C treatment group occupied this space indicating a greater variance amongst females from the 17°C population. The bursal valve shape from the 33°C treatment group were overall wider and longer than the 27°C group but this difference was not signficant. On PC1, increasingly thin bursal valves are associated with increasingly negative scores whereas positive scores were associated with wider valves. On PC2, increasingly long and wide bursal valves are associated with increasingly negative (respectively, positive) scores. The extreme positive values were associated with long and thin bursal valves. An NPMANOVA showed that this distribution was significant ($F_{2, 57}$ = 3.593, p = 0.015), whilst pairwise comparisons showed that the 17°C and 33°C populations were significantly different (p = 0.013). By constrast, neither comparisons between 17°C and 27°C, nor between 27°C and 33°C, were significantly different (p = 0.077 and p = 0.371, respectively).

2.4 Discussion

Developmental temperature affects female body size and the size and shape of the reproductive organs. Females grown at low temperatures were larger than females grown at higher temperatures. This is in accordance with Bergmann's rule which states that populations of a species are larger when found in colder environments, and are smaller when found in warmer environments (Kingsolver & Huey, 2008). An increase in body size with decreasing developmental temperature has been reported in a number of taxa including *C. maculatus* (Stillwell & Fox, 2007; Wu & Sun, 2012; Ghosh *et al*., 2013; Vasudeva, 2014).

Female body weight in *Callosobruchus chinensis* has been positively correlated with the width of the ovipositor, egg length, and egg width (Yanagi & Tuda, 2012). Vasudeva (2014) also found that developmental temperature affected the length and width of *C. maculatus* eggs; – females reared at 17°C and 33°C had larger eggs than females reared at 25°C and 27°C. Considering these findings, it is not surprising that developmental temperature also had a significant effect on the length, width and shape of the bursa copulatrix and the bursal valve. The bursa copulatrix and bursal valve of females that developed at a lower temperature were longer and narrower as well as being variable in shape, compared with females reared at 27°C or 33°C.

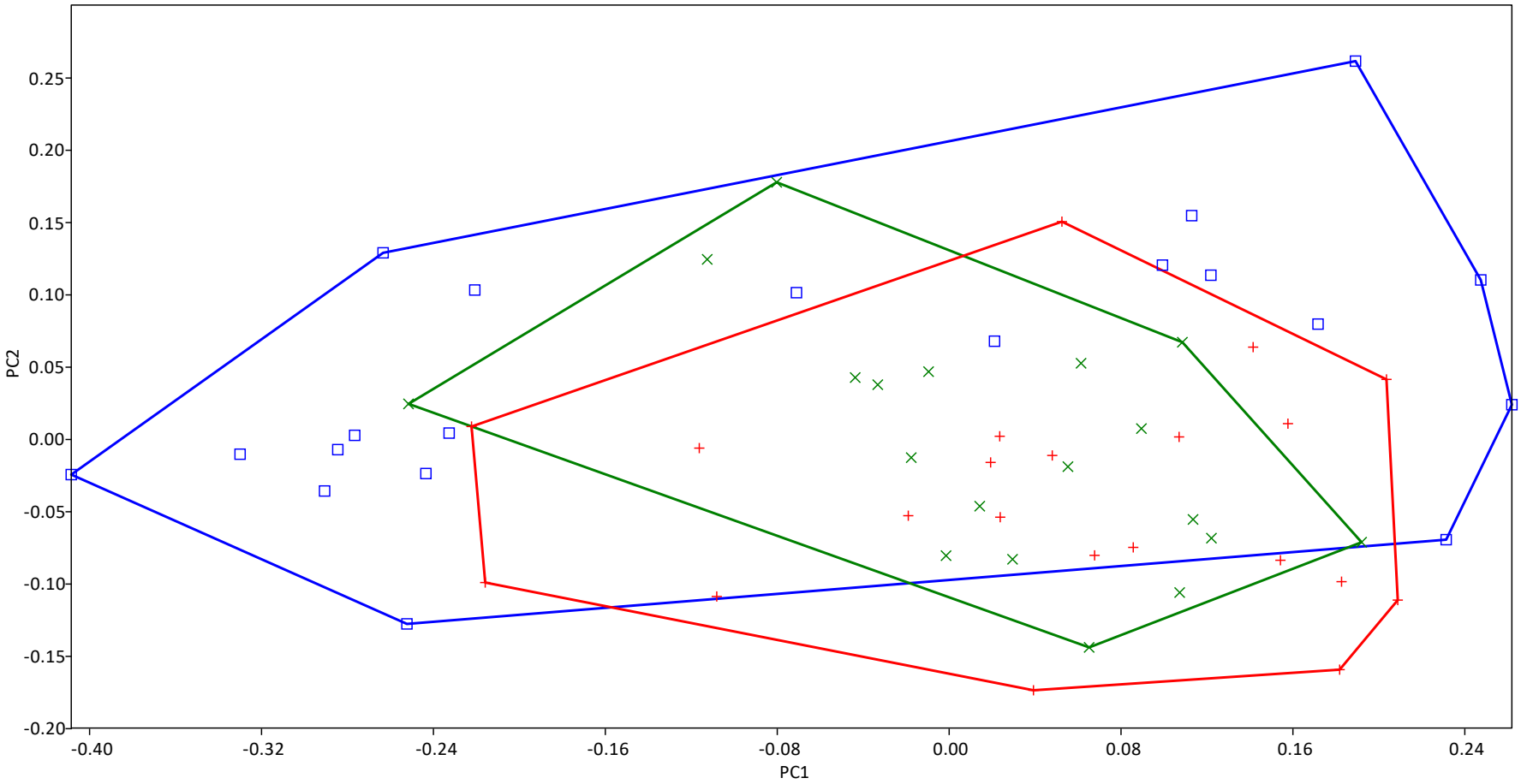


Figure 2.13. Convex hulls plot based on PC scores generated from the analysis of 60 outlines of bursal valves. Colours and lines indicate the three temperature groups in the set. (Blue = 17°C, Green = 27°C, and Red = 33°C).

Similar effects of developmental temperature on female reproductive architecture have been reported by Berger *et al*. (2011) who found that when yellow dung flies, (*Scatophaga stercoraria*) were reared at lower temperatures, female flies were more likely to have four spermatheca instead of the usual three.

Finding developmental plasticity in female reproductive architecture is important because there is good evidence that the female reproductive environment sets the rules by which sperm competition is played out (Eberhard, 1985, 1996; Wilson *et al*., 1997; Eady, 2001; Miller & Pitnick, 2002). Indeed, evidence of sperm-female trait coevolution points to the female reproductive environment being an important selection pressure on sperm evolution (Pitnick *et al*., 1999; Presgraves *et al*., 1999; Miller & Pitnick, 2002; Beese *et al*., 2009). Furthermore, Miller & Pitnick (2002) showed female reproductive architecture to influence the outcome of sperm competition in *D. melanogaster*: males with long sperm out-competed males with short sperm but only when sperm competition took place inside females selected to have long seminal receptacle lengths. Therefore, if female reproductive traits are plastic in relation to environmental conditions then there may be no optimal male trait (e.g. sperm length) because the environment in which the sperm operate is likely to change in relation to temporal and spatial variation in environmental conditions. In effect, if female reproductive architecture selects for male sperm traits (Miller & Pitnick 2002) then the addition of variation to the dimensions of the female reproductive environment, via environmental heterogeneity, will weaken selection for specific male (sperm) traits. An important consequence of such a scenario is that variation in female reproductive architecture would act to maintain genetic variation in high fitness reproductive traits providing a possible solution to the lek paradox (Kotiaho *et al*., 2007).

Demonstrating plasticity in female reproductive architecture is also important because there is good evidence that plastic responses to environmental variation can become genetically assimilated (Waddington, 1942; Pfennig *et al*., 2010). Essentially, this is a process whereby variation in a phenotype, which is triggered by environmental factors, becomes integrated into the genotype (Pigliucci *et al.*, 2006). Consequently, female reproductive traits may evolve in response to the physical environment (Beese *et al.,* 2009) altering the rules by which post-copulatory sexual selection is played out. This provides a possible solution to the question raised by Miller & Pitnick (2002): what selection pressures drive the evolution of female reproductive architecture?

Given that plasticity has been evidenced in male reproductive traits in response to temperature (Vasudeva *et al*., 2014) it was predicted that female reproductive traits would also respond plastically to fluctuations in developmental temperature. Females reared at 17^{o}C had the shortest spermathecal ducts, but differences between the treatment groups were not significant. This could be due to a small sample size in this part of the study, as previously reported (see Results 2.3.3). What is more likely, however is that the spermathecal duct absence from a number of 33^{o}C females was due to developmental temperature. A higher developmental temperature which results in quicker development (Stillwell & Fox, 2007) could result in more fragile reproductive traits, such as the spermathecal duct which may have been lost during the cleaning process. The thermal stress experienced by these females during development may have resulted in resources being diverted from reproductive development to somatic growth and development representing a developmental trade-off (Vasudeva *et al*., 2014). Further studies should look again at this trait, perhaps including individuals reared at a higher temperature such as 35^{o}C.

It is clear from the results that the responses of the female *C. maculatus* to thermal stress are plastic in nature. However, further work is needed to ascertain a more in depth picture of the mechanisms by which temperature affects female reproductive architecture and the consequences of variation in female reproductive architecture. There is evidence in *Drosophila* that genes important in the regulation of gonad morphogenesis are temperature sensitive (Schütt & Nöthiger) which could be an explanation as to the causal mechanism. Furthermore, thermal plasticity has also been reported in the lipid contents of cell membranes (Van Dooremalen *et al*., 2011) which could affect permeability of these membranes including the movement of hormones that are necessary for developmental regulation.

This research did not have the scope to investigate the spermatheca itself which is an important aspect of female reproductive morphology. It is possible that the spermatheca is affected by temperature as it has been in other species (Berger *et al*., 2011). A study focussing on the spermathecal size and shape and the effects of developmental temperature would add to the knowledge already gained in this area. Equally it would be interesting to discover the role of the bursal valve in the act of reproduction and what effects are experienced by the female as a result on the change of shape and dimensions of this trait.

Given developmental temperature affects female reproductive architecture, I now predict that developmental temperature will act either separately or in combination with (i.e. interact with) male developmental temperature to affect copulatory behaviour and the outcome of sperm competition. These two predictions form the basis of the next two chapters.

Chapter 3
The effect of developmental temperature on the copulatory behaviour of *Callosobruchus maculatus*

3.1 Introduction

Copulation is the act of genital coupling that functions in the transfer of sperm from the male reproductive tract to the female reproductive tract (Eberhard, 1985; Simmons, 2001). The duration of copulation varies enormously from a few seconds in the mosquito (*Aedes aegypti*; Spielman, 1964) and the stalk-eyed fly (*Cyrtodiopsis whitei*; Lorch *et al*., 1993), up to 6 hours for the two-spotted ladybird (*Adalia bipunctata;* de Jong *et al.*, 1998) and for several days in the stinkbug (*Nezara viridula;* Mitchell & Mau, 1969). This variation has typically been considered within a framework of sperm competition theory (Simmons, 2001; Pizzari & Parker, 2009) such that males vary the duration of copula in response to the risk of sperm competition (Simmons, 2001). For instance, in the soapberry bug (*Jadera haematoloma*), copulation can last from anywhere between 10 and 26 hours despite insemination occurring within 10 minutes of the start of copulation (Carroll, 1988). In this instance remaining in copula effectively guards against a rival mate copulating with the female. However, males also vary copulation duration in order to strategically adjust the volume of ejaculate in response to the presence of rival males, *i.e.* sperm competition risk (Kelly and Jennions, 2011).

Despite the fact that sperm competition has provided a useful functional framework for understanding copulatory behaviour, many studies overlook the role of the female in copulatory behaviour. Females may influence the duration of copulation in order to have control over which male fertilises her ova (cryptic female choice) or in response to sexual conflict (Crudgington & Siva-Jothy, 2001). For example, in hanging flies (*Bittacus apicalis*) copulation duration is related to nuptial gift size (Thornhill, 1976). When the nuptial gift was too small, females terminated copulation, whilst in cases where the nuptial gifts exceeded a threshold size ($18mm^2$), males terminated copulation. Thornhill's (1976) study suggests that male and female interests over the duration of copulation may be in conflict. Uncovering which sex ultimately controls this behaviour is a key challenge for behavioural ecologists. To unravel female and male influences over copulation duration, Krebs (1991) used strains of the fruit fly *Drosophila mojavensis* from different geographical regions and was able to partition variation in copulation duration between females and males. He found that both female and male population origin had a significant effect on copulation duration suggesting both sexes influence this behavioural trait.

Eady & Brown (MS) provided further evidence that copulation duration is a product of both female and male interests. Using *Callosobruchus maculatus*, Eady & Brown (MS) mated males to either 3 separate females or three times to the same female; similarly, they also mated females to either 3 separate males or three times to the same male. Copulation duration was non-repeatable when females copulated with three novel males or when males mated with three novel females. However, when females (or males) copulated repeatedly with the same individual copulation duration was highly repeatable, suggesting this behavioural trait to be a product of a male-female interaction.

In addition to female and male influences over copulation duration, the physical environment can also influence the duration of their behaviour. Temperature affects the rates and limits of physiological processes, especially in poikilotherms (Angiletta, 2009). These processes then limit or influence behavioural responses, including copulation duration. For example, when pairs of the predaceous mite (*Neoseiulus californicus*) were mated at one of four different temperatures (18^{o}C, 25^{o}C, 30^{o}C and 35^{o}C), Nguyen & Amano (2009) found that mating duration decreased with an increase in temperature. Similarly, when the beetle, *Callosobruchus chinensis* was mated at varying ambient temperatures (17^{o}C, 25^{o}C & 33^{o}C), individuals that mated at 17^{o}C took significantly longer to mate than individuals from the other temperature groups (Katsuki & Miyatake, 2009). A similar result was found by Vasudeva (2014) studying *C. maculatus*. In the rice-leaf roller (*Cnaphalocrocis medinalis*) Liao *et al.* (2014) found males subjected to heat shock (40^{o}C for 5 hours) mated less frequently than non-heat shocked males and when they did mate, the duration of copulation was significantly longer than that for untreated males. Male *C. maculatus* when subjected to heat shock of 50^{o}C for 50 minutes mated for significantly longer and had a much reduced ejaculate mass (van Lieshout *et al*., 2013). This calls into question the assumed direct relationship between copulation duration and ejaculate transfer (Kelly & Jennions, 2011). For example, Eady & Brown (MS) found the duration of copulation to increase with each successive male mating in *C. maculatus*. Males of this species are known to transfer smaller ejaculates (Fox *et al*., 1995) and transfer fewer sperm (Eady, 1995) with each successive mating. Furthermore, older male *C. maculatus* have been show to spend more time in copula but transfer smaller ejaculates than their younger counterparts (Fox *et al*., 1995; Ofuya, 1995), whilst Vasudeva *et al.* (2014) found that males exposed to thermal stress during development copulated for longer but transferred fewer sperm. Taken together these studies indicate copulation duration to be negatively associated with male quality in *C. maculatus* (Eady & Brown, MS), casting doubt on the general assumption that variation in copulation duration is a good proxy for variation in sperm transfer (Kelly & Jennions, 2011).

Given that copulation duration is most likely a product of a male and female interaction (Krebs, 1991; Wilson *et al.*, 1997; Simmons, 2001; Eady & Brown, MS), and that developmental temperature affects male (Vasudeva *et al*., 2014) and female reproductive anatomy and physiology (Berger *et al*., 2011; Chapter 2), it is predicted that the developmental temperature experienced by males and females will affect the duration of copulation in *C. maculatus*. Here three cultures of *C. maculatus* were reared 17°C, 27°C and 33°C and a factorial design deployed to study the effect of male and female developmental temperature and their interaction on the duration of copulation. The prediction being that both female and male developmental temperature will interact to affect the duration of copulation.

3.2 Materials and Methodology

3.2.1 Experimental design

Virgin males and females were collected from all three temperature groups (see Chapter 1) 0-24 hours post-eclosion, segregated and given 24 hours to acclimatise at 27°C. Beetles used in this experiment were never more than 48-hours post-eclosion. All matings took place in a temperature controlled insectary maintained at 27°C. A single virgin female was introduced to a single virgin male under a Petri dish, which was placed on a bench, and their behaviour observed. Females reared at 17°C, 27°C or 33°C were mated to males reared at 17°C, 27°C or 33°C in a fully factorial design. Typically, 30 pairings from each temperature group were carried out but only a small number of females only from the 17°C culture successfully copulated despite a large number of attempted matings (see Table 3.1). The duration of both the start-to-kick (Phase 1) and kick-to-end (Phase 2) phases (Eady, 1994b) were recorded using a stopwatch.

3.2.2 Data analysis

A very small proportion of the 17°C females actually copulated (8 out of 66 – 12%, Table 3.1) and so a full factorial analysis of copulation duration was not possible. Therefore, to assess the effect of female developmental temperature on copulation duration a one-way analysis of variance (ANOVA) was performed to assess the effect of female rearing temperature (17°C, 27°C and 33°C) on the duration of copulation when mated to 27°C males only. In the case of a significant result pairwise comparisons were made using a Tukey test. Female-male interaction effects were assessed in a two-way ANOVA that included only females cultured at 27°C and 33°C (because 17°C females failed to mate with 17°C and 33°C males) and 17°C, 27°C and 33°C males. All duration values were $\log_{10}$ transformed prior to analysis to normalise the data, and were analysed using IBM SPSS version 22.

Table 3.1: Overview of the number of attempted and successful matings for each female and male temperature treatment.

Female	Male	Number of pairings	Successful copulation
17°	17°	12	0
17°	27°	51	8
17°	33°	3	0
27°	17°	94	23
27°	27°	31	30
27°	33°	30	30
33°	17°	40	22
33°	27°	30	28
33°	33°	30	30

3.3 Results

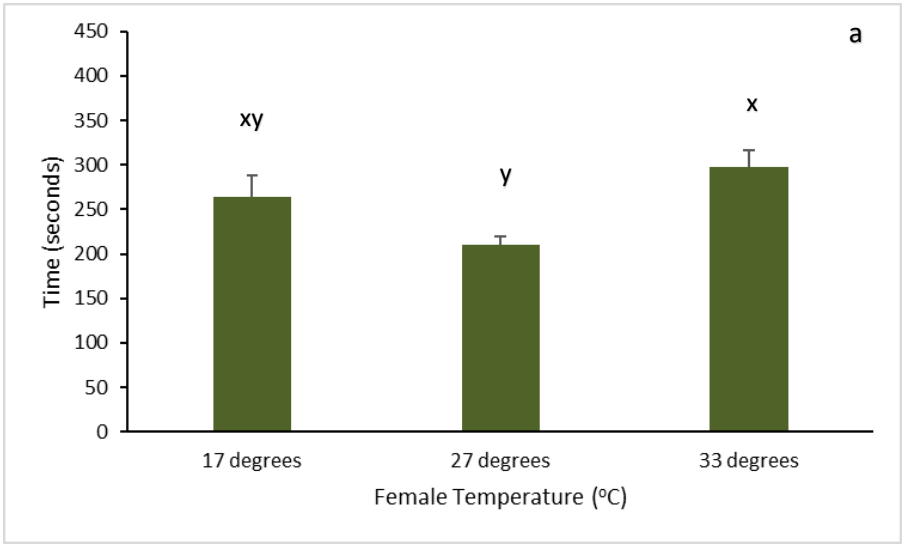
A total of approximately 320 male-female pairings were attempted. The majority of mating attempts involved males and females from the 17°C population (n = 200; see Table 3.1). This high number of attempted matings was necessary due to the reluctance of females and males from the 17°C population to mate.

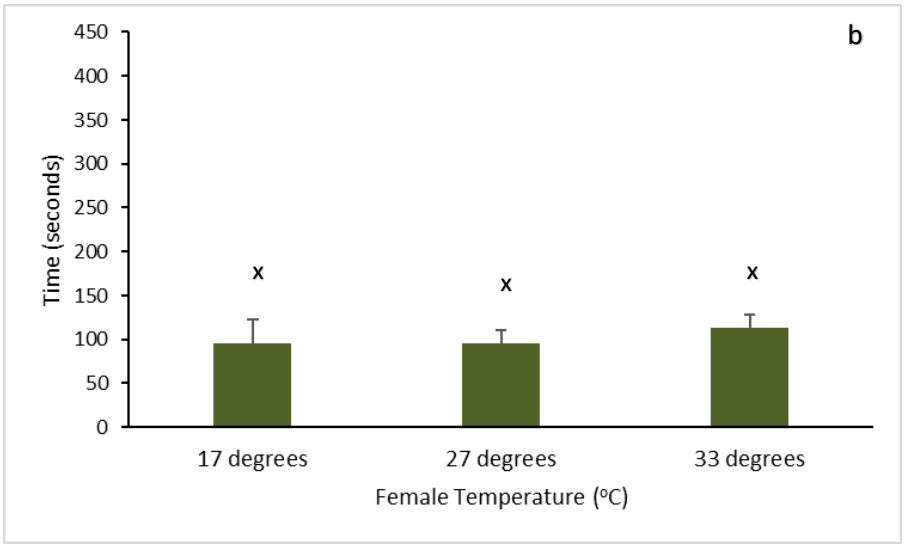
The start-to-kick phase of copulation lasted between 200 to 300 seconds depending on the temperature at which individuals were cultured (Figure 3.1a) with females from the 17°C and 33°C treatment group spending the most time in this phase. One-way ANOVA revealed a significant effect of female developmental temperature on the duration of the start-to-kick phase of copulation ($F_{2, 62} = 10.324$, $p < 0.0001$). Females reared at 27°C had the shortest duration (Figure 3.1a), which was significantly shorter than females at 33°C whilst all other pairwise comparisons were not significant (Figure 3.1a). The kick-to-end phase of copulation was shorter than the start-to-kick phase (~100s) for all female treatment groups but there was no significant effect of female developmental temperature on its duration ($F_{2, 62} = 0.403$, $p = 0.670$) (Figure 3.1b). Total copulation duration was similar in pattern to the start-to-kick phase with both the 17°C and 33°C females taking longer to complete copulation. Total copulation duration was shortest for those females reared at reared at 27°C and was significantly shorter than the females reared at 33°C but not 17°C (Figure 3.1c; $F_{2, 158} = 10.177$, $p < 0.0001$).

To examine the possibility of female-male interactions influencing copulation duration the 27°C and 33°C females were analysed in conjunction with the 17, 27 and 33°C males. Females reared at 27°C had a shorter duration of the start-to-kick phase of copulation that females reared at 33°C (Figure 3.2a) and the two-way ANOVA revealed that this difference was significant ($F_{1,160}$ = 20.21, p <0.0001). Male developmental temperature also affected this phase of copulation with males reared at 17°C taking significantly longer than males from the 27°C and 33°C treatments (*post-hoc* Tukey test, p = 0.032 and p = 0.014 respectively). There was no difference in the start-to-kick phase between males reared at 27°C and 33°C (*post-hoc* Tukey test, p = 0.96). There was no significant interaction between male and female developmental temperature on the duration of the start-to-kick phase of copulation ($F_{2,158}$ = 2.28, p = 0.106).

Female developmental temperature had no effect on the duration of the kick-to-end phase of copulation (Figure 3.2b; $F_{1,160}$ = 0.049, p = 0.826). By contrast, male developmental temperature significantly affected this second phase of copulation ($F_{2,160}$= 3.62, p = 0.029; Figure 3.2b) because males reared at 17°C experienced a longer kick-to-end phase. This was significantly longer than for than males reared at 27°C only (post-hoc Tukey test, p = 0.03). There was no significant interaction between male and female developmental temperature on the duration of the kick-to-end phase ($F_{2,158}$ = 1.81, p = 0.167).

Male and female developmental temperature did not interact to affect overall copulation duration (Figure 3.2c; $F_{2,158}$ = 0.876, p = 0.418). However, females reared at 33°C spent significantly longer in copula than their 27°C counterparts, particularly when mated with males from the 27°C and 33°C treatments (Figure 3.2c; $F_{1,160}$ = 15.282, p <0.0001). There was also a significant effect of male developmental temperature on overall copulation duration ($F_{2,160}$ = 9.71, p < 0.0001). Males reared at 17°C experienced a longer copulation than both 27°C and 33°C males (post-hoc Tukey test, p < 0.0001 for both).





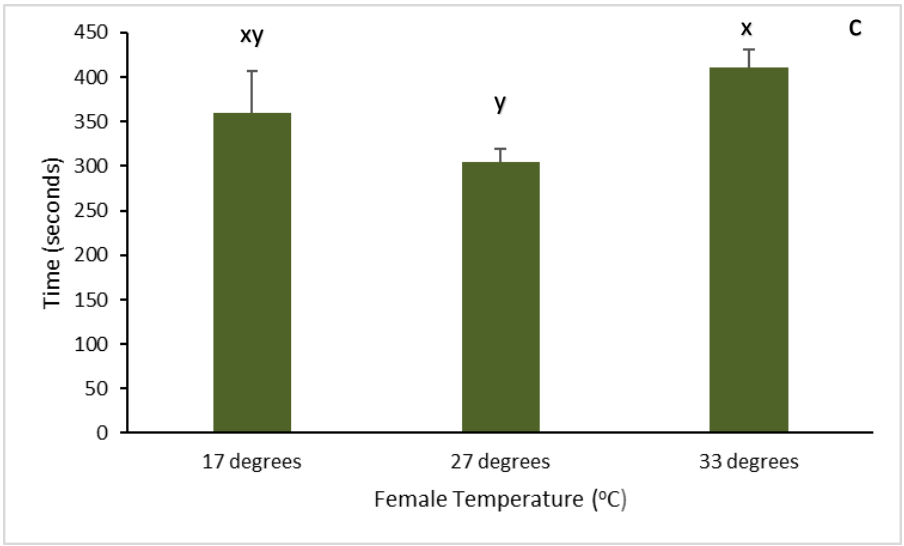


Figure 3.1: The mean (+SE) duration of a) start-to-kick, b) kick-to-end and c) total copulation for females reared at 17°C, 27°C and 33°C when mated to males reared at 27°C. Different letters above bars indicate significant differences between treatments as revealed by *post-hoc* Tukey test ($p < 0.05$).

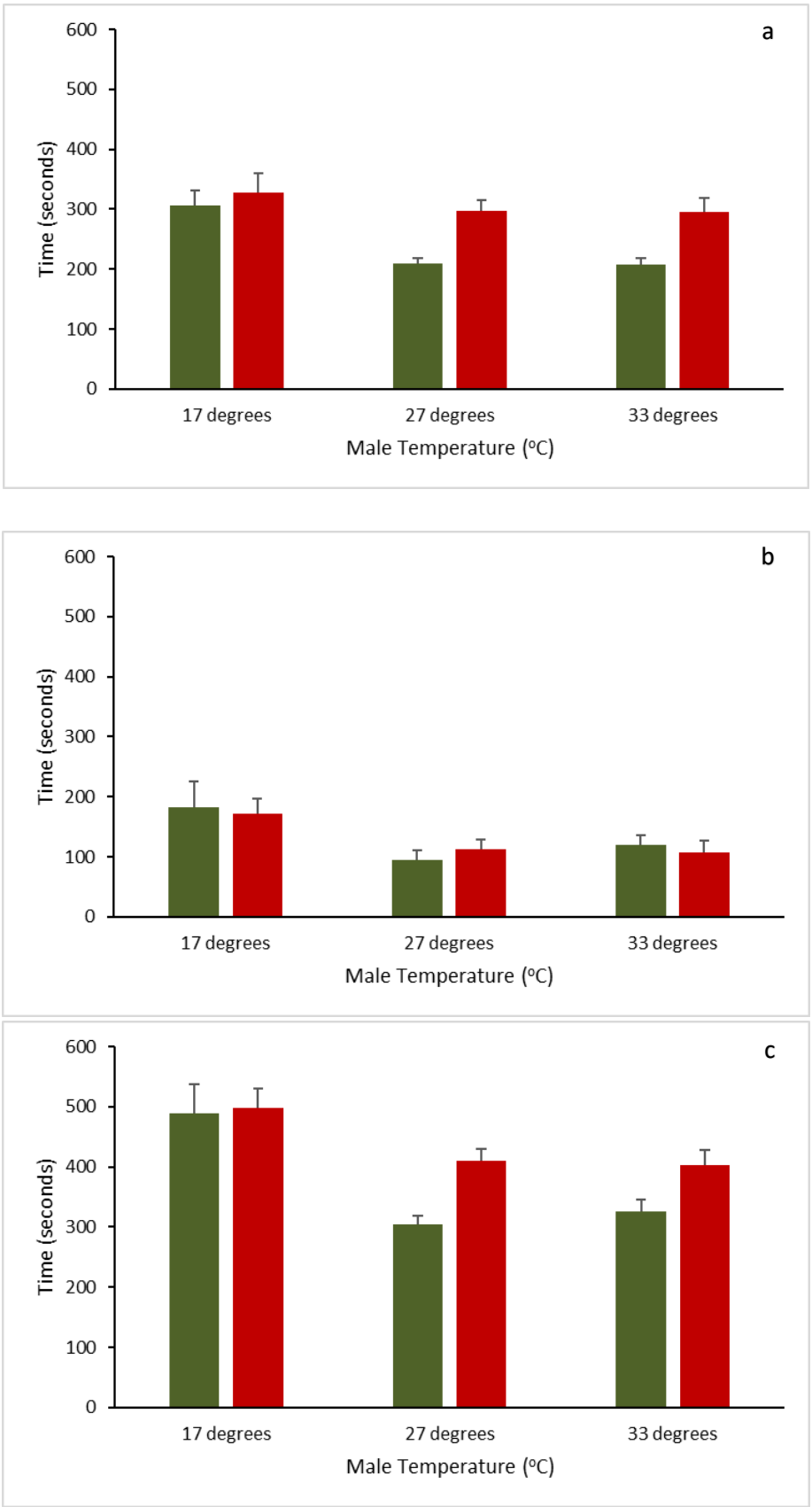


Figure 3.2: The mean (+SE) duration of a) start-to-kick, b) kick-to-end and c) total copulation for females from the 27ºC (green) and 33ºC population (red) when mated to males from 17ºC, 27ºC or 33ºC treatments.

3.4 Discussion

The results of this study indicated that the developmental temperature experienced by both males and females affected the duration of the start-to-kick phase and the total duration of copulation in *C. maculatus*. Females reared at the 'optimal' temperature (27°C) tended to have shorter durations of the start-to-kick phase and shorter total copulation durations. Male developmental temperature also impacted on copulatory behaviour: males reared at 17°C spent longer in the first phase and second phase of copulation and subsequently copulated for longer than males reared at 33°C and 27°C.

Why developmental temperature should affect the copulation behaviour of females is unclear. As reported earlier (see Chapter 2), developmental temperature is known to affect female reproductive architecture. The bursa copulatrix of females reared at 17°C tended to be longer and thinner than those reared at 27°C or 33°C whilst those reared at 33°C tended to have smaller bursas than those reared at 27°C. The bursa copulatrix could be a central component in the female regulation of copulatory behaviour. Male's deposit their spermatophore in the bursa copulatrix and it is thought that stretch receptors in the wall of this organ signal to the female that ejaculate transfer is complete and to thus terminate copulation (Stringer *et al*., 1985). In lepidoptera, experimental bursal inflation (via injection of silicone oil) resulted in afferent impulses from the bursal nerves (Sugawara, 1979). Therefore, it is possible that stretch receptors in the wall of the bursa provides the female with information about ejaculate transfer that could be used to inform decisions about remaining (or otherwise) in copula. Indeed, Eady and Brown (MS) show that when males transfer smaller ejaculates the start-to-kick phase is longer. So a possible mechanism is that developmental temperature affects bursal morphology (and most likely physiology) which subsequently affects the afferent signals sent by bursal nerves to the females' brain affecting her copulatory behaviour. Another related possibility is that alteration of female reproductive architecture influences (disrupts) the male's ability to transfer an ejaculate. This could slow bursal filling and extend the first phase (start-to-kick) of copulation. Finally, it's possible that females in poor condition (*i.e*. those reared under sub-optimal temperatures) were physiologically stressed and thus less able to exact control over copulation duration.

Male developmental temperature also had an effect on the duration of the start-to-kick phase and total copulation duration; males reared at 17°C had longer durations irrespective of the female developmental temperature. This result confirms the results of Vasudeva (2014) who found that males mated at 17°C and males reared at 17°C (and mated at an ambient temperature of 27°C) took longer to complete the start-to-kick phase than those mated or

reared at higher temperatures. This fits with the notion that copulation duration is negatively related to ejaculate size in this species (Eady & Brown, MS). Males reared at 17°C transferred fewest sperm (Vasudeva *et al*., 2014) and copulated for longer, although Edvardsson & Canal (2006) reported larger ejaculate transfer when the duration of the kick-to-end phase was longer. However, in this study, males reared at 17°C are possibly less fit than males reared at the higher temperatures. This would result in these males transferring less ejaculate (Eady, 1994b; Fox *et al*., 1995; Vasudeva, 2014) resulting in reduced stimulation of bursal stretch receptors and consequently fewer afferent impulses from the bursal nerves (Stringer *et al*., 1985). The upshot of this hypothesised sequence of events would be a reduction in the likelihood of females initiating the termination of copulation (i.e. the kicking phase).

Female developmental temperature had no effect on the duration of the kick-to-end phase of copulation but male developmental temperature did. It took longer for females to dislodge males from the 17°C group than from males reared at 27°C and 33°C during the kick-to-end phase. This is in contrast to Vasudeva (2014) who found no effect of male developmental temperature on the duration of the kick-to-end phase (although the males reared at 17°C did have, albeit non-significant, a longer kick-to-end duration). Why male developmental temperature affected the duration of the kick-to end phase is open to speculation. Males reared at 17°C are larger than those reared at 27°C or 33°C (Vasudeva, 2014). Thus, it is possible that 17°C males also had larger intromittent organs than males reared at 27°C or 33°C, resulting in females requiring more time to dislodge the 17°C males during the kicking phase. However, Hotzy *et al*. (2012) report spine length in male *C. maculatus* to have no detectable effect on copulation duration, although they did not relate spine length to the duration of the kick-to-end phase of copulation. Additionally, male genital width and not spine length *per se* could be the important driver of variation in the second phase of copulation. Future research into this area should perhaps focus on how temperature affects male genitalia, particularly the spinal adaptations.

The question as to why females from the 17°C treatment group were so unlikely to mate is interesting. Previous studies have found *Callosobruchus* spp. to be less likely to mate at temperatures as low as 17°C (Katsuki & Miyatake, 2009) and when they did mate, the duration was considerably longer than beetles that mated at warmer temperatures (Katsuki & Miyatake, 2009; Vasudeva, 2014). However, all matings in this study took place at 27°C and thus ambient temperature can be ruled out as the cause of female reluctance to mate in the present study. Being reared at 17°C may delay sexual maturity in this group resulting in adults not being capable of mating upon eclosion even though the norm in *C. maculatus* is emerging adults ready to mate

(Vasudeva *et al*., 2014). The unwillingness of females to mate from the 17°C treatment group, could be in part due to bursal stretch receptors sending the message that the bursa copulatrix is not being capable of receiving a spermatophore from the male, due to developmental pathology. This is not dissimilar to a female being unwilling to remate immediately after copulation.

Eady (1995) suggested that the volume of seminal fluid in the bursa copulatrix determines how receptive the female is to re-mating because when a large ejaculate was received by the female she was less likely to remate. This is further supported by research from Edvardsson & Canal (2006) who attempted to remate females immediately after their first mating and found only a very low number of females willing to remate. When preparing specimens for investigation using geometric morphometrics (Chapter 2) it was apparent that several individuals could not be used due to a dark-beige-brown mass present in the bursa copulatrix of females reared at 17°C. To my knowledge this has not been previously reported. One possible explanation for this is that the gastro-intestinal tract opening is very close to the opening of the atrium which leads into the bursa copulatrix (Mukerji & Hakim Bhuya, 1937). Developmental stress could have resulted in incomplete separation of these two systems resulting in females being unable to copulate. Future studies could look into this feature in individuals reared at lower temperatures.

In the wild, *C. maculatus* are found in bean stores in tropical areas such as West Africa and South Asia. Bean stores create their own micro-climate in that the centre of the store can be considerably warmer than the outer areas, often experiencing up to 8°C in difference (Appleby & Credland, 2007). This study and that of Vasudeva (2014) have shown that a variation in temperature of just 6°C can significantly affect both the growth and development of female and male primary reproductive traits respectively. Furthermore, these developmental differences appear to directly translate into differences in female and male copulatory behaviour. The next question is, do these differences in female and male developmental temperatures also affect the outcome of sperm competition?

Chapter 4
The effect of developmental temperature on sperm competition in the seed beetle *Callosobruchus maculatus*

4.1 Introduction

Sperm competition in insects occurs when the sperm from two or more males compete within the female's genital tract to fertilise her ova (Parker 1970; Simmons, 2001; Birkhead & Pizzari, 2002; Snook 2005). Sperm competition tends to select for males that inseminate more sperm (Parker, 1998; Simmons, 2001; Gage & Morrow, 2003) although across bush-cricket taxa, a greater ejaculate volume is associated with a lower level of polyandry (Vahed, 2006). However, comparative studies in multiple taxa have demonstrated evolutionary increases in testes size in response to increased likelihood of sperm competition risk (Stockley *et al*., 1997; Byrne *et al*., 2002; Vahed *et al*., 2011). In addition, there is good evidence that within species, males increase ejaculatory expenditure when the risk of sperm competition is high (Kelly & Jennions, 2011). Sperm competition also tends to select for better sperm quality. Rowe & Pruett-Jones (2011) found a positive association between the level of sperm competition and sperm quality as measured by three different phenotypic traits, motility, percentage of live sperm and sperm morphology, in the Australian passerine family, Maluridae. However, the outcome of sperm competition is not solely dependent on male ejaculatory characteristics. Eberhard (1985; 1996) was amongst the first to champion the view that male performance in sperm competition was strongly dependent on female reproductive behaviour, anatomy and physiology. Such male-female interactions were described as cryptic female choice. For example, studying *Drosophila melanogaster*, Miller and Pitnick (2002) found males selected to have long sperm achieved greater fertilisation success than males selected for shorter sperm, but only when the competition took pace in females selected to have long, as opposed to short, seminal receptacle lengths. Such male-female interactions are also evident from comparative studies that report co-evolution between sperm length and dimensions of the female reproductive tract (Pitnick *et al*., 1999; Miller & Pitnick, 2002).

Of interest, recent studies have shown both male and female reproductive traits to be influenced by the physical environment experienced during development, specifically, temperature. Blackenhorn & Hellriegel (2002) studying yellow dung flies (*Scathophaga stercoraria*) were the first to show that sperm length varied with developmental temperature. Subsequent studies by Minoretti *et al*. (2013) on the land snail *Arianta arbustorum*, Breckels & Neff (2013) on the guppy *Poecilia reticulata* and Vasudeva *et al*. (2014) on *Callosobruchus*

maculatus have also shown developmental temperature to affect sperm size. In *C. maculatus*, males reared at either low or high temperatures produced shorter sperm than those reared at the 'optimal' temperature of 27°C (Vasudeva *et al*., 2014).

Developmental temperature has also been shown to influence female reproductive anatomy. Berger *et al*. (2011) working with yellow dung flies, found that higher developmental temperature resulted in a change in female phenotype with a greater incidence of the 4 spermathecae phenotype as opposed to the usual three spermathecae being present. Given that development temperature affects male (Vasudeva *et al*., 2014) and female (Berger *et al*., 2011; see Chapter 2) reproductive traits and that both can influence the outcome of sperm competition (Miller & Pitnick, 2002), it is likely that altering developmental temperature of both male and female will affect the outcome of sperm competition. To date only a few studies have looked at the effects of developmental temperature on the outcome of sperm competition and, of interest, both are on *C. maculatus*. Van Lieshout *et al*. (2013) subjected *C. maculatus* pupae from populations that were either inbred or outbred to a heat shock of 50°C for 50 minutes and compared them with non-heat shocked controls. Being from either the inbred or outbred group had no impact on sperm precedence. However, males from the heat shock group fertilised only half the number of offspring as non-heat shocked males. The other study was Vasudeva *et al*. (2014) who found males reared at either hot or cold temperature extremes performed less well in sperm competition than males reared at intermediate temperatures. Unfortunately, it is difficult to ascertain what mechanism(s) operate to produce this result as developmental temperature affected sperm number, copulatory behaviour and sperm length (Vasudeva, 2014), plus it might have affected the quantity and quality of seminal proteins within the ejaculate.

To study sperm competition experimentally virgin females are sequentially mated to two or more males (Fricke *et al*., 2010) and the offspring identified by a phenotypic trait. In *C. maculatus* this is achieved by using wild-type (WT) and melanistic morphs of the beetles (black-type: BT). The outcome of sperm competition is typically measured as the proportion of eggs fertilised by the sperm of the last male to mate, typically termed P_2 (Boorman & Parker 1976; Eady, 1991; Simmons, 2001; Eady, 2010). By contrast, P_1 is the proportion of eggs fertilised by the first male to mate with the female.

To date (and to the best of my knowledge) no-one has simultaneously examined how developmental temperature experienced by males and females interact to influence the outcome of sperm competition. Here females and males were cultured at different temperatures and through a factorial design the effects of female and male developmental

temperature on P_1 and P_2 were determined. In light of previous studies (Vasudeva *et al*., 2014), males reared at the two temperature extremes were predicted to perform less well in sperm competition than males reared at the intermediate (optimal) temperature. By contrast, because female developmental temperature affects the arena in which sperm competition is played out (chapter 2), I predicted female developmental temperature to affect the outcome of sperm competition.

4.2 Materials and Methodology

Black-type (BT) cultures were initiated at 17°C, 27°C and 33°C (see Chapter 1), alongside a control population of tan beetles (wild-type: WT) at 27°C. Rearing and adult collection methods were as described in Chapter 1.

The genetic marker technique (Eady, 1991) was used to determine the effects of developmental temperature on sperm precedence. All matings (see Table 4.1) were carried out under the same conditions as those described in Chapter 3. To establish the effect of development temperature on P_2, virgin BT females reared at 17°C, 27°C and 33°C, were mated to individual virgin WT males and 24-48 hours later, remated to a virgin BT male, reared at 17°C, 27°C and 33°C. To determine the effect of development temperature on P_1, virgin BT females were first mated to virgin BT males followed by virgin WT males (as above). Approximately 30 pairings from each temperature group were carried out (see Table 4.2). After the first mating, males were removed and the females individually placed in a small petri dish with approximately 50 moth beans and allowed to oviposit. Twenty-four hours after the first mating, a second mating was carried out with either a virgin BT or WT male. If this second attempted mating was unsuccessful then another attempted mating ensued no more than 48 hours after the first mating. Following copulation, the male was removed and the female placed on approximately 100 fresh moth beans to lay eggs until natural death. Both sets of eggs from each female were kept in an incubator at 27°C until all viable offspring had emerged. These offspring were then stored in a -20°C freezer for later analysis.

All offspring were subsequently counted and grouped either as being offspring of a Black Type (BT) or Wild Type (WT) male (see Chapter 1). A very small number of 17°C females successfully mated despite a large number of attempted matings (Table 4.2). Even fewer 17°C females copulated a second time. Consequently, the 17°C females were excluded from the sperm competition experiment (see Results). Data were analysed using a GLM (generalized linear

model) binomial logistic model using the statistical package S-Plus. The full P2 model was initially fitted and contained the independent variables; male developmental temperature, female developmental temperature and their interaction. Significance of main and interaction effects was determined via F-tests (to compensate for over-dispersion) following model simplification via a series of stepwise deletion tests, starting with the interaction term. The critical probability for retention of a factor or interaction term was 5% (Crawley, 2002).

Table 4.1: An overview of the mating crosses used for both the behavioural and sperm competition experiments

Dependent Variable	**Female**		**1st Male**		**2nd Male**
P_1	♀ BT (17^0, 27° or 33°)	x	♂ BT (17^0, 27° or 33°)	x	♂ WT 27^0
P_2	♀ BT (17^0, 27° or 33°)	x	♂ WT 27^0	x	♂ BT (17^0, 27° or 33°)

Table 4.2: Overview of the number of attempted and successful matings for each female population

Female	1st male	2nd male	Attempted no. of pairings *	Successful 1st mating	Successful 2nd mating
17°	17°	WT	12	0	0
17°	27°	WT	51	8	4
17°	33°	WT	3^	0	0
17°	WT	17°	44	7	0
17°	WT	27°	1^	1	1
17°	WT	33°	0^	0	0
27°	17°	WT	94	23	23
27°	27°	WT		30	29
27°	33°	WT		30	23
27°	WT	17°	281	N/A	23
27°	WT	27°		30	22
27°	WT	33°		29	28
33°	17°	WT	40	22	22
33°	27°	WT		28	24
33°	33°	WT		30	28
33°	WT	17°	254	N/A	16
33°	WT	27°		30	27
33°	WT	33°		30	26

N/A = Times not recorded for first mate in this instance
* = Only relevant to those mating with a 17°C male or female
^ = No/few attempts as decision made to exclude 17°C females

4.3 Results

4.3.1 Sperm offence – P_2 (proportion of offspring fertilised by the second male to mate)

Males reared at 17°C tended to perform best (in terms of P_2) when mating with 27°C females whilst males reared at 27 and 33 degrees tended to achieve higher P_2 when mated to 33°C females. The GLM (generalized linear model) binomial logistic model on all these data revealed a significant male*female interaction on P_2: Δ deviance = 88.06, F = 3.61, p = 0.03. However, 45% and 76% of the data associated with 17°C males mated to 27°C and 33°C females revealed $P_2 = 0$. Thus, it is possible these males were infertile and transferred no sperm during copulation.

Therefore, the P_2 data were re-analysed using just males and females reared at 27°C or 33°C. The GLM revealed no significant male*female interaction on P_2 (Δ deviance = 2.16, F = 0.23, p = 0.63) and, as such, this term was removed from the model. The final model (for 27°C or 33°C males and females) revealed a significant effect of male development temperature (Δ deviance = 39.8, F = 4.39, p = 0.039) and a significant effect of female development temperature (Δ deviance = 43.2, F = 4.75 p = 0.032) on P_2. Males reared at 27°C achieved slightly higher P_2 than males reared at 33°C whilst females reared at 33°C were associated with higher P_2 values than females reared at 27°C (Figure 4.1).

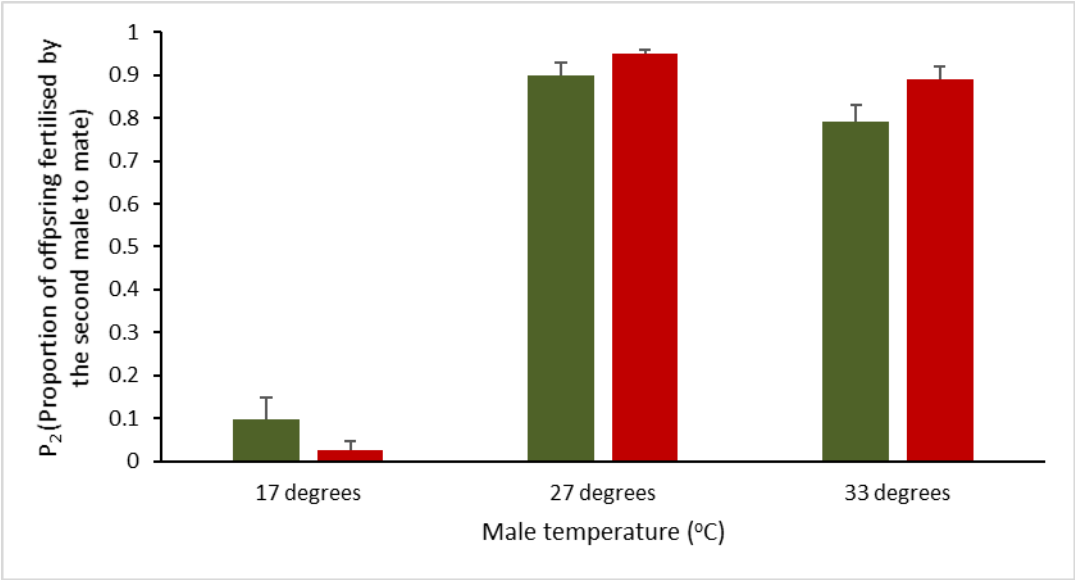


Figure 4.1 Mean (+SE) P_2 for males from all three treatment groups crossed with 27°C (green) and 33°C females (red).

4.3.2 Sperm defence – P_1 (proportion of offspring fertilised by the first male to mate)

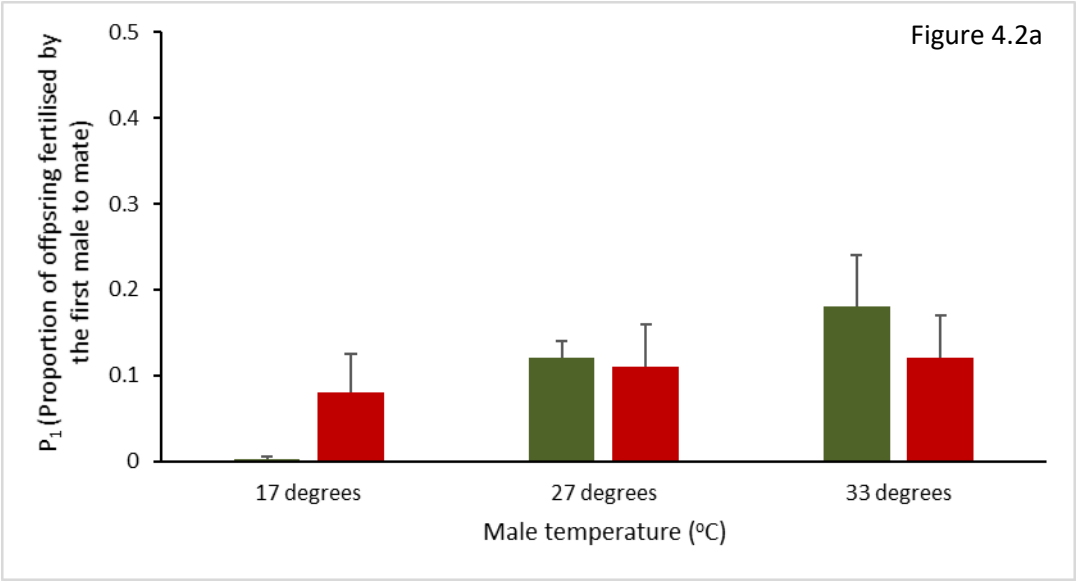
Males reared at 27°C and 33°C tended to achieve higher P_1-values than males reared at 17°C, especially when mated to females that were reared at 27°C (Figure 4.2a). A GLM revealed no significant male*female interaction P_1 (Δ deviance = 108, F = 2.92, p = 0.057). This term was subsequently removed from the model. The final model recorded a significant effect of male development temperature only on P_1 (Δ deviance = 356, F = 7.88, p = 0.0005). However, a high number of 17°C males failed to fertilise any eggs when mated to a 27°C (82%) or 33°C (55%) females (Figure 4.2b). Only 4% of males reared at 33°C failed to fertilise any offspring when mated to a female from either temperature group and none of the 27°C males were infertile when mated to either a 27°C or 33°C female. Therefore, the P_1 data were re-analysed using only males that were proven to be fertile. The GLM revealed no significant male*female interaction on P_1 (Δ deviance = 63.1, F = 1.6, p = 0.2). Thus, this term was removed from the model. The final model recorded a non-significant effect of either male development temperature (Δ deviance = 34.9, F = 0.88, p = 0.41) or female development temperature (Δ deviance = 3.84, F = 0.19, p = 0.66) on P_1.

4.4 Discussion

4.4.1 Sperm offence – P_2

Males from the 17°C treatment group did not perform well during sperm competition with P_2 values averaging < 0.1. Males from the 27°C and 33°C treatment groups displayed P_2 values more typical of *C. maculatus* (Eady, 1991) being in the range 0.79 to 0.95. The results also provide some evidence that male and female development temperature interact to affect P_2, although the interaction might be a reflection of the fact that many of the males reared at 17°C were in fact not transferring fertile sperm (and therefore not technically entering into sperm competition).

However, despite this the results do point to male development temperature (see also Vasudeva *et al.*, 2014) and female development temperature affecting P_2: males reared at 27°C tended to out-perform those males reared at 17°C and 33°C, while females reared at 33°C tended to be associated with higher P_2 values than females reared at 27°C. That P_2 is a product of a male-female interaction was shown by Wilson *et al.* (1997) in *C. maculatus* and by Miller & Pitnick (2002) in *D. melanogaster*. In Miller & Pitnick's (2002) study, males with long sperm outcompeted males with short sperm but only when the competition took place inside females selected to have long seminal receptacle length.



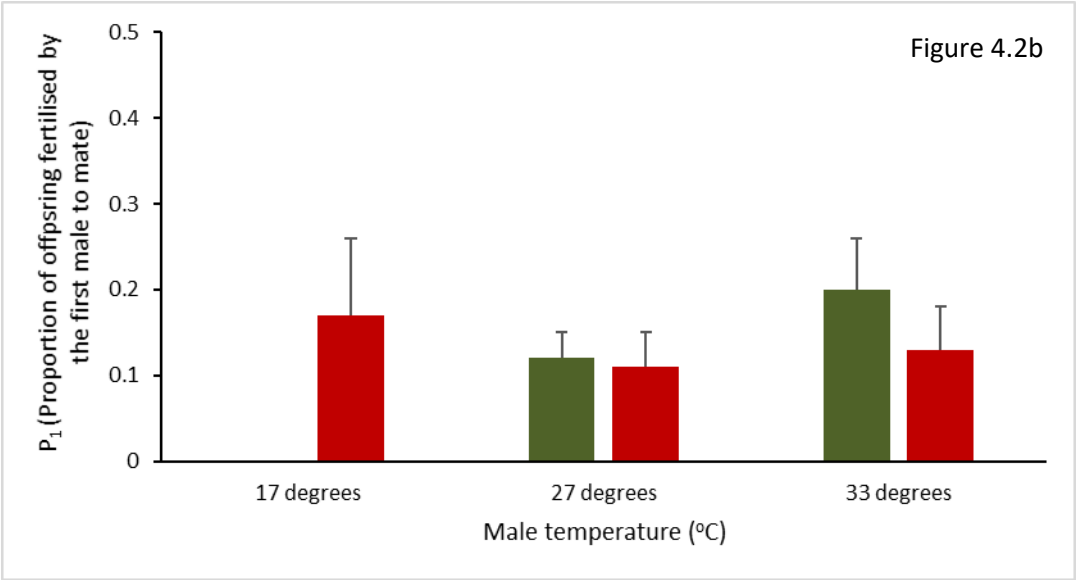


Figure 4.2 a & b. Mean (+SE) of P_1 for males from all three treatment groups crossed with 27°C (green) and 33°C females (red). Figure 4.2b excludes copulations that resulted in no fertile eggs being laid in the 24h between copulations as it is possible these males were infertile.

Males reared at 27°C had the highest P_2 values as also reported by Vasudeva *et al.* (2014). These males also had longer sperm than males reared at 17°C or 33°C and inseminated slightly more sperm (Vasudeva *et al*., 2014). This makes it difficult to identify the mechanisms by which these males achieve the highest P_2 but it is unlikely to be a result of greater number of sperm transferred because Eady (1995) found experimental manipulation of sperm number to have little impact on P_2. Males from the 27°C treatment copulated for a similar length of time to males reared at 33°C but for shorter than males reared at 17°C. Thus, there is no obvious link between P_2 and copulation duration (see also Eady, 1994a).

Females reared at 33°C, as opposed to 27°C, tended to be associated with higher P_2 values. Developmental temperature does affect female reproductive architecture in *C. maculatus* (see Chapter 2) and other species (Berger *et al*., 2011; de Guzman *et al*., 2015). Thus, it is possible that plastic responses of the female reproductive tract to environmental conditions alters the arena in which sperm competition is played out (Eberhard, 1996; Miller & Pitnick, 2002) so affecting P_2. However, this result could also be a consequence of 33°C females experiencing greater environmental stress during development such that upon eclosion to adult the females are in some way less fit than the 27°C females. Through being weakened by developmental stress it is possible the 33°C females have fewer resources to invest into the maintenance of stored sperm. Thus, at the time of the second mating the 33°C females have fewer first male sperm in their sperm storage organs. Further study into the relationship between female condition and P_2 and female development temperature and sperm storage utilisation would be required to test this concept.

4.4.2 Sperm defence – P_1

Male development temperature had a significant effect on P_1 (see also Vasudeva *et al*., 2014). Males reared at 17°C tended to have lower P_1 values than males reared at 27°C or 33°C. This is probably due to the 17°C males transferring fewer and/or smaller sperm (Vasudeva, 2014) although given the high number of 17°C male matings that resulted in no offspring prior to the second mating by the female, it is also possible a proportion of the 17°C males transferred no sperm. In heterospecific sperm competition trials between *C. maculatus* males and *C. subinnotatus*, the sperm of *C. subinnotatus* males outcompeted those of *C. maculatus*. Although this result could be due to several factors, it was noted that *C. subinnotatus* had the longest sperm (Rugman-Jones & Eady, 2008). Thus, it is possible that the 17°C males did poorly in sperm competition as a result of having small sperm. There was no effect of female development temperature on P_1 although any effect from females reared at the lowest temperature (17°C) could not be ascertained as so few females in this group mated to 17°C males. Indeed, only 14% of females reared at 17°C mated at all. This is considerably lower than the 85% of females reared at 30°C remating (Eady, 1991), which is probably lower than the percentage of 27°C females mating for the first time (personal observation). Considering the hesitant reproductive behaviour from females reared at 17°C (see Chapter 2), it is clear that developmental temperature has a profound effect on female reproductive anatomy and behaviour.

In the parasitoid wasp (*Anisopteromalus calandrae*), a 6-degree Celsius increase in temperature from the norm (temperature increased at the point where the testes were formed but not the seminal vesicles) led to a halving of sperm numbers, while an 8-degree increase resulted in 10 times less sperm (Nguyen *et al*., 2013). However, Nguyen *et al.* (2013) did not investigate the effects of temperatures lower than the norm on sperm production. Sperm numbers alone do not predict successful fertilisation because there are a host of sperm traits, for example sperm size, speed, mobility etc., that could have an effect on sperm precedence (Snook, 2005). Hotzy & Arnqvist (2009) reported that male *C. maculatus* with longer genital spines sired a higher proportion of offspring when they were the last male to mate (higher P_2). They proposed that this resulted from the transfer of seminal fluid proteins through the wall of the female reproductive tract and into the female haemolymph, subsequently altering how the female stored and/or utilised the sperm of males with longer genital spines. It is possible developmental temperature had an effect on the expression of male genital spines in the present study. Such speculation is reasonable given developmental temperature affects female reproductive architecture (see Chapter 2) and male reproductive traits, including absolute and relative testes size in *C. maculatus* (Vasudeva, 2014).

To conclude, this is the first study (to my knowledge) to demonstrate that female developmental temperature affects the outcome of sperm competition. This is important because in nature, poikilothermic animals are likely to experience a wide range of developmental environments (Appleby & Credland, 2007; Kvist *et al.*, 2013; Penttilä *et al*., 2013). Such variation in environmental conditions could affect the development and expression of female reproductive architecture, which in turn could affect which sperm are utilised at fertilization and thus the outcome of sperm competition. In turn, the developmental environments experienced by females could affect the intensity and direction of selection acting on male primary reproductive traits, providing a mechanism by which variation in high fitness male traits (i.e. sperm morphology) is maintained (Howie *et al*., 2013; Johnston *et al*., 2013).

Chapter 5
General Conclusion

This body of work set out to determine what effect development temperature had on the reproductive behaviour of both female and male *Callosobruchus maculatus* along with assessing the effect of developmental temperature on female reproductive architecture. When a female mates with two males during a single reproductive episode, sperm competition ensues. The outcome of this competition is difficult to predict because it depends in part on the behaviour, morphology and physiology of the 1st male to mate, the 2nd male to mate and the female herself. These traits could also interact between any two of the protagonists or between all three players. The situation is further complicated when we consider developmental plasticity in these traits.

To date few studies have documented developmental plasticity in reproductive traits (beyond those associated with sperm competition risk) and fewer still have examined how plasticity in both male and female traits interact to influence the outcome of sperm competition. The study presented here aimed to redress this situation and demonstrated that developmental temperature affected the plastic expression of female reproductive architecture, copulatory behaviour and the outcome of sperm competition.

I argue that this has important implications for the evolution of primary reproductive traits on two fronts. Firstly, increasing variation in female reproductive architecture, a trait known to co-evolve with sperm length, via environmental (temperature) perturbation, will reduce the intensity of selection on males to produce a single optimal sperm phenotype. Effectively maintaining genetic variation in this high fitness trait: a problem that has spawned numerous theoretical and empirical studies into the phenomenon known as the lek paradox (Kotiaho *et al*., 2007; Kotiaho *et al*., 2008; Madden & Whiteside, 2013). Secondly, there is growing awareness that trait plasticity might actually speed the process of evolutionary change rather than the traditional view that it would slow the process down (Nijhout, 1999; Pfennig *et al*., 2010; Fitzpatrick, 2012). Primary reproductive traits are considered to have evolved both rapidly and divergently, yet the role of plasticity in this process has rarely been considered. Should an environmental perturbation persist (global warming) then a plastic response, say in the reproductive architecture of the female, could become genetically assimilated. Given the strong evidence of sperm-female co-evolution in a number of taxa, it is then possible that changes to the female reproductive environment could send sperm off on different evolutionary trajectories. Clearly at present these are speculative scenarios and thus I suggest the next

obvious step would be to see if long-term exposure to environmental stress (temperature) results in the accelerated evolution of male and female primary reproductive traits.

References

Angilletta, M.J.Jr. (2009) *Thermal adaptation: a theoretical and empirical synthesis*. Oxford: Oxford University Press.

Anten-Houston, Matthew V. (2015) *The interrelationships between egg shape and pelvis morphology in birds*. MSc. University of Lincoln.

Appleby, J.H. and Credland, P.F. (2007) The role of temperature and larval crowding in morph determination in a tropical beetle, *Callosobruchus subinnotatus*. *Journal of Insect Physiology*, 53, 983-993.

Arnqvist, G. (1998) Comparative evidence for the evolution of genitalia by sexual selection. *Nature*, 393, 784-786.

Baer, B., Schmid-Hempel, P., Hoeg, J.T. and Boomsma, J.J. (2003) Sperm length, sperm storage and mating system characteristics in bumblebees. *Insectes Sociaux*, 50, 101-108.

Beck, C.W. and Blumer, L.S. (2014) A handbook on bean beetles, *Callosobruchus maculatus*. Atlanta: Emory University and Morehouse College. Available from: http://beanbeetles.org/handbook/handbook.pdf [accessed 29 March 2015].

Beese, K., Armbruster, G.F.J, Beier, K. and Baur, B. (2009) Evolution of female sperm-storage organs in the carrefour of stylommatophoran gastropods. *Journal of Zoological Systematics and Evolutionary Research*, 47, 49-60.

Berger, D., Bauerfeind, S.S., Blanckenhorn, W.U. and Schafer, M.A. (2011) High temperatures reveal cryptic genetic variation in a polymorphic female sperm storage organ. *Evolution*, 65, 2830-2842.

Birkhead, T.R. (1998) Cryptic female choice: criteria for establishing female sperm choice. *Evolution*, 52, 1212-1218.

Birkhead, T.R. & Møller, A.P. (1998) *Sperm competition and sexual selection*. San Diego: Academic Press.

Birkhead, T.R. and Pizzari, T. (2002) Postcopulatory sexual selection. *Nature Reviews. Genetics*, 3, 262-273.

Blackenhorn, W.U. and Hellriegel, B. (2002) Against Bergmann's rule: fly sperm size increases with temperature. *Ecology Letters*, 5, 7-10.

Bolles, K.L. and Begg, G.A. (2000) Distinction between silver hake (*Merluccius bilinearis*) stocks in U.S. waters of the northwest Atlantic based on whole otolith morphometrics. *Fishery Bulletin*, 98, 451-462.

Boorman, E. and Parker, G.A. (1976) Sperm (ejaculate) competition in *Drosophila melanogaster* and the reproductive value of females to males in relation to female age and mating status. *Ecological Entomology*, 1, 145-155.

Boughman, J.W. (2002) How sensory drive can promote speciation. *Trends in Ecology and Evolution*, 17 (12) 571-577.

Breckels, R.D. and Neff, B.D. (2013) The effects of elevated temperature on the sexual traits, immunology and survivorship of a tropical ectotherm. *Journal of Experimental Biology*, 216, 2654-2664.

Brown, D.V. and Eady, P.E. (2001) Functional incompatibility between the fertilization systems of two allopatric populations of *Callosobruchus maculatus* (Coleoptera: Bruchidae). *Evolution*, 55 (11) 2257-2262.

Byrne, P.G., Roberts, J.D. and Simmons, L.W. (2002) Sperm competition selects for increased testes mass in Australian frogs. *Journal of Evolutionary Biology,* 15, 347-355.

Carroll, S.P. (1988) Contrasts in reproductive ecology between temperate and tropical populations of *Jadera haematoloma*, a mate-guarding Hemipteran (Rhopalidae). *Annals of the Entomological Society of America*, 81, 54-63.

Clark, A.G., Begun, D.J. and Prout, T. (1999) Female x male interactions in *Drosophila* sperm competition. *Science*, 51, 251-265.

Clarke, A. (2003) Costs and consequences of evolutionary temperature adaptation. *Trends in Ecology and Evolution*, 18, 573-581.

Crawley, M.J. (2002) *Statistical computing: an introduction to data analysis using S-Plus*. Chichester. Wiley.

Crudgington, H.S. & Siva-Jothy, M.T. (2000) Genital damage, kicking and early death. *Nature*, 407, 855-856.

Darwin, C. (1871) *The descent of man and selection in relation to sex*. Reprinted. New York: Modern Library.

de Guzman, L.I., Rinderer, T.E. and Frake, A.M. (2015) The effects of diet, mating duration, female to male ratios, and temperature on ovary activation, mating success, and fecundity of *Aethina tunida*. *Apidologie*, 46, 326-336.

de Jong, G. and van der Have, T.M. (2009) Temperature dependence of development rate, growth rate and size: from biophysics to adaptation. In Whitman, D.W. and Ananthakrishnan, T.N. (eds.) *Phenotypic plasticity of insects: mechanisms and consequences*. Enfield: Science Publishers, 523-588.

de Jong, P.W., Brakefield, P.M. and Geernick, B.P. (1998) The effect of female mating history on sperm precedence in the two-spot ladybird *Adalia bipunctata* (Coleoptera: Coccinellidae). *Behavioral Ecology*, 9, 559-565.

Deeming, D.C. and Ruta, M. (2014) Egg shape changes at the theropod – bird transition, and a morphometric study of amniote eggs. *Royal Society Open Science*, 1, 140311.

Dybas, L.K. and Dybas, H.S. (1981) Coadaptation and taxonomic differentiation of sperm and spermatheca in featherwing beetles. *Evolution*, 35, 168-174.

Eady, P.E. (1991) Sperm competition in *Callosobruchus maculatus* (Coleoptera: Bruchidae): a comparison of two methods used to estimate paternity. *Ecological Entomology*, 16, 45-53.

Eady, P.E. (1994a) Intraspecific variation in sperm precedence in the bruchid beetle *Callosobruchus maculatus*. *Ecological Entomology*, 19, 11-16.

Eady, P.E. (1994b) Sperm transfer and storage in relation to sperm competition in *Callosobruchus maculatus*. *Behavioural Ecology and Sociobiology*, 35, 123-129.

Eady, P.E. (1995) Why do male *Callosobruchus maculatus* beetles inseminate so many sperm? *Behavioural Ecology and Sociobiology*, 36, 25-32.

Eady, P.E. (2001) Postcopulatory, prezygotic reproductive isolation. *Journal of Zoology*, 253, 47-52.

Eady, P.E. (2010) Postcopulatory sexual selection in the Coleoptera: mechanisms and consequences. In: J.L. Leonard and A. Cordoba-Aguilar (eds.) *The evolution of primary sexual characters in animals*. Oxford: Oxford University Press, 353-378.

Eady, P.E. and Brown, D.V. (MS) It takes two to tango: the (un)repeatability of copulation duration.

Eady, P.E. (2015) Personal communication.

Eberhard, W.G. (1985) *Sexual selection and animal genitalia*. Cambridge: Harvard University Press.

Eberhard, W.G. (1996) *Female control: sexual selection by cryptic female choice*. New Jersey: Princeton University Press.

Eberhard, W.G. (2008) Static allometry and animal genitalia. *Evolution*, 63, 48-66.

Eberhard, W.G. (2010) Rapid divergent evolution of genitalia: theory and data updated. In J.L. Leonard and A. Cordoba-Aguilar (eds.) *The evolution of primary sexual characters in animals*. Oxford: Oxford University Press, 40-78.

Edvardsson, M. and Canal, D. (2006) The effects of copulation duration in the bruchid beetle *Callosobruchus maculatus*. *Behavioral Ecology*, 17, 430-434.

Fox, C.W., Hickman, D.L., Raleigh, E.L. and Mousseau, T.A. (1995) Paternal investment in a seed beetle (Coleoptera: Bruchidae): influence of male size, age, and mating history. *Annals of the Entomological Society of America*, 88, 100-103.

Fricke, C., Andersson, C. and Arnqvist, G. (2010) Natural selection hampers divergence of reproductive traits in a seed beetle. *Journal of Evolutionary Biology*, 23, 1857-1867.

Friedrich, F., Matsumura, Y., Pohl, H., Bai, M., Hornschmeyer, T. and Beutel, R.G. (2013) Insect morphology in the age of phylogenomics: innovative techniques and its future role in systematics. *Entomological Science*, 17, 1-24.

Gage, M.J.G. and Morrow, E.H. (2003) Experimental evidence for the evolution of numerous, tiny sperm via sperm competition. *Current Biology*, 13, 754-757.

Ghiselin, M.T. (2010) The distinction between primary and secondary sexual characters. In J.L. Leonard and A. Cordoba-Aguilar (eds.) *The evolution of primary sexual characters in animals*. Oxford: Oxford University Press, 9-14.

Ghosh, S.M., Testa, N.D. and Shingleton, A.W. (2013) Temperature-size rule is mediated by thermal plasticity of critical size in *Drosophila melanogaster*. *Proceedings of the Royal Society of London B: Biological Sciences*, 280, 20130174.

Hammer, Ø., Harper, D.A.T. and Rya, P.D. (2001) PAST: Paleontological Statistics software package for education and data analysis. *Palaeontologia Electronica* 4, 9.

Harris, W.E., Moore, A.J. and Moore, P.J. (2007) Variation in sperm size within and between ejaculates in a cockroach. *Functional Ecology*, 21, 598-602.

Hotzy, C. and Arnqvist, G. (2009) Sperm competition favors harmful males in seed beetles. *Current Biology*, 19, 404-407.

Hotzy, C., Polak, M., Rönn, J.L. and Arnqvist, G. (2012) Phenotypic engineering unveils the function of genital morphology. *Current Biology*, 22, 2258-2261.

Houck, L.D. and Verrell, P.A. (2010) Evolution of primary sexual characters in amphibians. In J.L. Leonard and A. Cordoba-Aguilar (eds.) *The evolution of primary sexual characters in animals*. Oxford: Oxford University, 409-424.

Howie, J., Pomiankowski, A. and Cotton, A.J. (2013) Evolution: sex or survival. *Current Biology*, 23, R1041-R1043.

Hunter, F.M. and Birkhead, T.R. (2002) Sperm viability and sperm competition in insects. *Current Biology*, 12, 121-123.

Janzen, F.J. (1995) Experimental evidence for the evolutionary significance of temperature dependent sex determination. *Evolution*, 49, 864-873.

Jensen, R. J. (2003) The conundrum of morphometrics. *Taxon*, 52, 663-671.

Johnston, S.E., Gratten, J., Berenos, C., Pilkington, J.G., Clutton-Brock, T.H., Pemberton, J.M. and Slate, J. (2013) Life history trade-offs at a single locus maintain sexually selected genetic variation. *Nature*, 502, 93-96.

Katsuki, M. and Miyatake, T. (2009) Effects of temperature on mating duration, sperm transfer and remating frequency in *Callosobruchus chinensis*. *Journal of Insect Physiology*, 55, 113-116.

Keating, J.P., Brophy, D., Officer, R.A. and Mullins, E. (2014) Otolith shape analysis of blue whiting suggests a complex stock structure at their spawning grounds in the Northeast Atlantic. *Fisheries Research*, 157, 1-6.

Kelly, C.D. and Jennions, M.D. (2011) Sexual selection and sperm quantity: meta-analyses of strategic ejaculation. *Biological Reviews*.

Kingsolver, J.G. and Huey, R.B. (2008) Size, temperature, and fitness: three rules. *Evolutionary Ecology Research*, 10, 251-268.

Klingenberg, C.P. (2011) MorphoJ: an integrated software package for geometric morphometrics. *Molecular Ecology Resources*, 11, 353-357.

Kotiaho, J.S., LeBas, N.R., Puurtinen, M. and Tomkins, J.L. (2007) On the resolution of the lek paradox. *Trends in Ecology and Evolution*, 23, 1-3.

Kotiaho, J.S., LeBas, N.R., Puurtinen, M. and Tomkins, J.L. (2008) On female choice, heterozygosity and the lek paradox. *Animal Behaviour*, 75 e1-e3.

Krebs, J.R. (1991) Function and genetics of long versus short copulations in the cactophilic fruit-fly, *Drosophila mojavensis* (Diptera: Drosophilidae). *Journal of Insect Behavior*, 4, 221-233.

Kvist, J., What, C.W., Kallioniemi, E., Saastamoinen, M., Hanski, I. and Frilander, M.J. (2013) Temperature treatments during larval development reveal extensive heritable and plastic variation in gene expression and life history traits. *Molecular Ecology*, 22, 602-619.

Leonard, J.L. (2010) Introduction: celebrating and understanding reproductive diversity. In J.L. Leonard and A. Cordoba-Aguilar (eds.) *The evolution of primary sexual characters in animals*. Oxford: Oxford University Press, 1-5.

Liao, H.J., Qian, Q. and Liu, X.D. (2014) Heat shock suppresses mating and sperm transfer in the rice leaf folder *Cnaphalocrocis medinalis*. *Bulletin of Entomological Research*, 104, 383-392.

Lorch, P.D., Wilkinson, G.S. and Reillo, P.R. (1993) Copulation duration and sperm precedence in the stalk-eyed fly *Cyrtodiopsis whitei* (Diptera: Diopsidae). *Behavioral Ecology and Sociobiology*, 32, 303-311.

Marshall, J. (2007) Rapid evolution of spermathecal duct length in the *Allonemobius socius* complex of crickets: species, population and Wolbachia effects. *PLoS One*, 2, e720.

Miller, G.T. and Pitnick, C. (2002) Sperm-Female coevolution in *Drosophila*. *Science*, 298, 1230-1233.

Minoretti, N., Stoll, P. and Baur, B. (2013) Heritability of sperm length and adult shell size in the land snail *Arianta arbustorum* (Linnaeus, 1758). *Journal of Molluscan Studies*, 79, 218-224.

Mitchell, W.C. and Mau, R.F.L. (1969) Sexual activity and longevity of the southern green stink bug *Nezara viridula*. *Annals of the Entomological Society of America*, 62, 1246-1247.

Møller, A.P. (1998) Sperm competition and sexual selection. In: T.R. Birkhead and A.P. Møller (eds.) *Sperm competition and sexual selection*. San Diego: Academic Press, 55-90.

Mukerji, D. and Hakim Bhuya, M. A. (1937) Reproductive system of the bruchid beetles *Bruchus quadrimaculatus* fabr. *Bruchus* (*Callosobruchus*) *chinensis* L., (Bruchidae-Coleoptera). *Journal of Morphology*, 16, 175-221.

Nguyen, T.P.T. and Amano, H. (2009) Mating duration and egg production of the predaceous mite *Neoseiulus californicus* (Acari: Phytoseiidae) vary with temperature. *Journal of Asia-Pacific Entomology*, 12, 297-299.

Nguyen, T.M., Bressac, C. and Chevrier, C. (2013) Heat stress affects male reproduction in a parasitoid wasp. *Journal of Insect Physiology*, 59, 248-254.

Ofuya, T.I. (1995) Multiple mating and its consequences in males of *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae). *Journal of Stored Products Research*, 31, 71-75.

Parker, G.A. (1970) Sperm competition and its evolutionary consequences in the insects. *Biological Reviews*, 45, 525-567.

Parker, G.A. (1998) Sperm competition and the evolution of ejaculates: towards a theory base. In Birkhead, T.R. & Møller, A.P. (eds.) *Sperm competition and sexual selection*. San Diego: Academic Press, 3-54.

Penttilä, A., Slade, E.M., Simojoki, A., Riutta, T., Minkkinen, K. and Roslin, T. (2013) Quantifying beetle-mediated effects on gas fluxes from dung pats. *PLoS ONE*, 8, e71454.

Pérez-Crespo, M., Pintado, B. and Gutiérrez-Adán, A. (2008) Scrotal heat stress effects on sperm viability, sperm DNA integrity, and the offspring sext ration in mice. *Molecular Reproduction and Development*, 75, 40-47.

Pfennig, D.W., Wund, M.A., Snell-Rood, E.C., Cruickshank, T., Schlichting, C.D. and Moczek, A.P. (2010) Phenotypic plasticity's impacts on diversification and speciation. *Trends in Ecology and Evolution*, 25, 459-467.

Pigliucci, M., Murrne, C.J. and Schlichting, C. D. (2006) Phenotypic plasticity and evolution by genetic assimilation. *Journal of Experimental Biology*, 209, 2362-2367.

Pitnick, S., Markow, T. and Spicer, G.S. (1999) Evolution of multiple kinds of female sperm-storage organs in *Drosophila*. *Evolution*, 53, 1804-1822.

Pitnick, S., Wolfner, M.F. and Suarez, S.S. (2009) Ejaculate-female and sperm-female interactions. In T.R. Birkhead, D.J. Hosken and S. Pitnick (eds.) *Sperm Biology: an evolutionary perspective*. Amsterdam: Academic Press, 247-304.

Pizzari, T. and Parker, G.A. (2009) Sperm competition and sperm phenotype. In T.R. Birkhead, D.J. Hosken and S. Pitnick (eds.) *Sperm Biology: an evolutionary perspective*. Amsterdam: Academic Press, 207-245.

Polly, P.D. (2012) Geometric morphometrics: an introduction. Available from http://www.indiana.edu/~g562/Lectures/Lecture%201%20-%20Introduction%20to%20Geometric%20Morphometrics.pdf [accessed 07 February 2016].

Presgraves, D.C., Baker, R.H. and Wilkinson, G.S. (1999) Coevolution of sperm and female reproductive tract morphology in stalk-eyed flies. *Proceedings of the Royal Society London B*, 266, 1041-1047.

Rasband, W. (2015) *ImageJ 1.50b*. National Institutes of Health, USA. Available from http://imagej.nih.gov/ij [accessed 14 January 2016].

Rice, W.R. (1996) Sexually antagonistic male adaptation triggered by experimental arrest of female evolution. *Nature*, 381, 232-234.

Rohlf, F.J. (2005) tpsDig, digitize landmarks and outlines, Version 2.17. Department of Ecology and Evolution, State University of New York at Stony Brook. Available from http://life.bio.sunysb.edu/morph [accessed 16 January 2016].

Rowe, M. and Pruett-Jones, S. (2011) Sperm competition selects for sperm quantity and quality in the Australian Maluridae. *PLoS ONE*, 6, e15720.

Rugman-Jones, P.F. and Eady, P.E. (2008) Co-evolution of male and female reproductive traits across the Bruchidae (Coleoptera). *Functional Ecology*, 22, 880-886.

Schäfer, M.A., Berger, D., Jochmann, R., Blackenhorn, W.U. and Burrière, L.F. (2013) The developmental plasticity and functional significance of an additional sperm storage compartment in female yellow dung flies. *Functional Ecology*, 37, 1392-1402.

Schütt, C. and Nöthiger, R. (2000) Structure, function and evolution of sex-determining systems in Dipteran insects. *Development*, 127, 667-677.

Simmons, L.W. (2001) *Sperm competition and its evolutionary consequences in the insects*. Princeton: Princeton University Press.

Simmons, L.W. and Kotiaho, J.S. (2007) Quantitative genetic correlation between trait and preferences supports a sexually selected sperm process. *Proceedings of the National Academy of Sciences*, 104, 16604-16608.

Simmons, L.W. (2014) Sexual selection and genital evolution. *Australian Journal of Entomology*, 53, 1-17.

Smith, R.L. (1984) *Sperm competition and the evolution of animal mating systems*. New York: Academic Press.

Snook, R.R. (2005) Sperm in competition: not playing by numbers. *Trends in Ecology and Evolution*, 20, 46-53.

Spielman, A. (1964) The mechanics of copulation in *Aedes aegypti*. *Biological Bulletin*, 127, 324-344.

Stilwell, R.C. and Fox, C.W. (2007) Environmental effects on sexual size dimorphism of a seed-feeding beetle. *Oecologia*, 153, 273-280.

Stockley, P., Gage, M.J.G., Parker, G.A. and Møller, A.P. (1997) Sperm competition in fishes: the evolution of testis size and ejaculate characteristics. *American Naturalist*, 149, 933-954.

Stringer, I.A.N., Giebultowicz, J.M. and Riddiford, L.M. (1985) Role of the bursa copulatrix in egg maturation and reproductive behaviour of the tobacco hawk moth, *Manduca sexta*. *International Journal of Invertebrate Reproduction and Development*, 8, 83-91.

Sugawara, T. (1979) Stretch reception in the bursa copulatrix of the butterfly, *Pieris rapae crucivora*, and its role in behaviour. *Journal of Comparative Physiology A*, 130, 191-199.

Thornhill, R. (1973) Sexual selection and nuptial feeding behaviour in *Bittacus apicalis* (Insecta: Mecoptera). *American Naturalist*, 110, 529-548.

Timms, R. (1998) Size-independent effects of larval host on adult fitness in *Callosobruchus maculatus*. *Ecological Entomology*, 23, 480-483.

Vahed, K. (2006) Larger ejaculate volumes are associated with a lower degree of polyandry across bushcricket taxa. *Proceedings of the Royal Society B*, 273, 2387-2394.

Vahed, K. (2015) Cryptic female choice in crickets and relatives (Orthoptera: Ensifera). In Peretti, A.V. and Aisenberg, A. (eds.) *Cryptic female choice in arthropods*. Switzerland: Springer International Publishing, 285-324.

Vahed, K., Parker, D.J. and Gilbert, J.D.J. (2011) Larger testes are associated with a higher level of polyandry, but a smaller ejaculate volume, across bushcricket species (Tettigoniidae). *Biology Letters*, 7, 261-264.

Van Dooremalen, C., Koekkoek, J. and Ellers, J. (2011) Temperature-induced plasticity in membrane and storage lipid composition: thermal reaction norms across five difference temperatures. *Journal of Insect Physiology*, 57, 285-291.

Van Lieshout, E., Tomkins, J.L. and Simmons, L.W. (2013) Heat stress but not inbreeding affects offensive sperm competitiveness in *Callosobruchus maculatus*. *Ecology and Evolution*, 3, 2859-2866.

Vasudeva, R. (2014) *The influence of developmental temperature on sperm form and function in Callosobruchus maculatus*. PhD. University of Lincoln.

Vasudeva, R., Deeming, D.C. and Eady, P.E. (2014) Developmental temperature affects the expression of ejaculatory traits and the outcome of sperm competition in *Callosobruchus maculatus*. *Journal of Evolutionary Biology*, 27, 1811-1818.

Viscosi, V. and Cardini, A. (2011) Leaf morphology, taxonomy and geometric morphometrics: a simplified protocol for beginners. *PLoS ONE*, 6, e25630.

Waddington, C.H. (1942) Canalization of development and the inheritance of acquired characters. *Nature*, 150, 563-565.

Whitman, D.W. & Agrawal, A.A. (2009) What is phenotypic plasticity and why is it important? In Whitman, D.W. and Ananthakrishnan, T.N. (eds.) *Phenotypic plasticity of Insects: mechanisms and consequences*. Enfield: Science Publishers, 1-63.

Wilson, K. and Hill, L. (1989) Factors affecting egg maturation in the bean weevil *Callosobruchus maculatus*. *Physiological Entomology*, 14, 115-126.

Wilson, N., Tubman, S.C., Eady, P.E. and Robertson, G.W. (1997) Female genotype affects male success in sperm competition. *Proceedings of the Royal Society B*, 264, 1491-1495.

Wu, X.W. and Sun, S.C. (2012) Artificial warming advances egg-laying and decreases larval size in the dung beetles *Aphodius erraticus* (Coleoptera: Scarabaeidae) in a Tibetan alpine meadow. Annales Zoologici Fennici, 49, 174-180.

Yanagi, S. and Tuda, M. (2012) Female size constrains egg size via the influence of reproductive organ size and resource storage in the seed beetle *Callosobruchus chinensis*. *Journal of Insect Physiology*, 50, 1432-1437.

Zelditch, L., Swiderski, D. L. and Sheets, H. D. (2012) *Geometric morphometrics for biologists: a primer* [ebook]. Second edition. Amsterdam: Academic Press.